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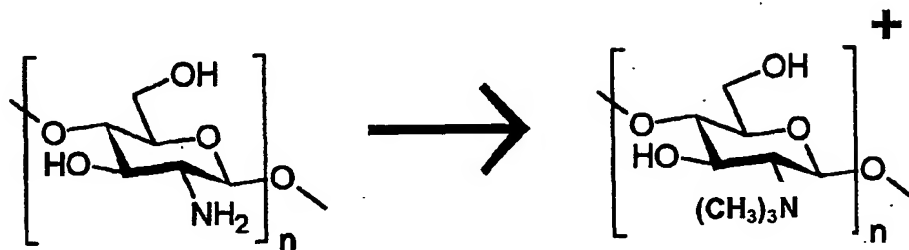
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(54) Title: **MUCOSAL MENINGOCOCCAL VACCINES**



(57) Abstract: The invention provides immunogenic compositions for mucosal delivery comprising capsular saccharides from at least two of serogroups A, C, W135 and Y of *N.meningitidis*. It is preferred that the capsular saccharides in the compositions of the invention are conjugated to carrier protein(s) and/or are oligosaccharides. Conjugated oligosaccharide antigens are particularly preferred. The invention also provides immunogenic compositions comprising (a) a capsular saccharide antigen from serogroup C of *N.meningitidis*, and (b) a chitosan adjuvant. The composition preferably comprises (c) one or more further antigens and/or (d) one or more further adjuvants. The compositions are particularly suitable for mucosal delivery, including intranasal delivery. The use of chitosan and/or detoxified ADP-ribosylating toxin adjuvants enhances anti-meningococcal mucosal immune responses and can shift the Th1/Th2 bias of the responses.

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MUCOSAL MENINGOCOCCAL VACCINES

All documents cited herein are incorporated by reference in their entirety.

TECHNICAL FIELD

This invention is in the field of vaccines, particularly against meningococcal infection and disease.

5 BACKGROUND ART

Neisseria meningitidis is a Gram-negative human pathogen [e.g. see Chapter 28 of ref. 1] which causes bacterial meningitis. It is closely related to *N.gonorrhoeae*, although one feature that clearly differentiates meningococcus is the presence of a polysaccharide capsule that is present in all pathogenic meningococci.

- 10 Based on the organism's capsular polysaccharide, twelve serogroups of *N.meningitidis* have been identified (A, B, C, H, I, K, L, 29E, W135, X, Y and Z). Group A is most common cause of epidemic disease in sub-Saharan Africa. Serogroups B & C are responsible for the vast majority of cases in developed countries, with the remaining cases being caused by serogroups W135 & Y.

As well as being used for classification, the capsular polysaccharide has been used for vaccination.

- 15 An injectable tetravalent vaccine of capsular polysaccharides from serogroups A, C, Y & W135 has been known for many years [2, 3] and is licensed for human use. Although effective in adolescents and adults, it induces a poor immune response and short duration of protection and cannot be used in infants [e.g. 4]. The polysaccharides in this vaccine are unconjugated and are present at a 1:1:1:1 weight ratio [5]. MENCEVAX ACWY™ and MENOMUNE™ both contain 50µg of each purified
20 polysaccharide once reconstituted from their lyophilised forms.

- Conjugated serogroup C oligosaccharides have been approved for human use [e.g. Menjugate™; ref. 6]. There remains, however, a need for improvements in conjugate vaccines against serogroups A, W135 and Y, and in their manufacture. That need is addressed by the products, processes and uses disclosed in reference 8, but there remains scope for further modifications and improvements,
25 particularly in relation to delivery and formulation.

DISCLOSURE OF THE INVENTION

The invention provides an immunogenic composition, comprising (a) a capsular saccharide antigen from serogroup C of *N.meningitidis*, and (b) a chitosan adjuvant. The composition preferably comprises (c) one or more further antigens and/or (d) one or more further adjuvants.

- 30 The invention also provides an immunogenic composition for mucosal delivery, comprising capsular saccharides from at least two of serogroups A, C, W135 and Y of *N.meningitidis*.

It is preferred that the capsular saccharides in the compositions of the invention are conjugated to carrier protein(s) and/or are oligosaccharides. Conjugated oligosaccharide antigens (Figure 1) are particularly preferred.

Capsular saccharide antigen from serogroup C meningococcus

The capsular saccharide of serogroup C of *N.meningitidis* has been widely used as an antigen. The active ingredient of Menjugate™, for instance, is an oligosaccharide fragment of the capsular polysaccharide, conjugated to CRM₁₉₇ carrier protein.

- 5 Where a composition of the invention includes a capsular saccharide antigen from serogroup C of *N.meningitidis*, it is thus preferred to use an oligosaccharide fragment of the capsular polysaccharide and/or to conjugate the saccharide antigen to a carrier protein. Particularly preferred MenC saccharide antigens are disclosed in references 6 & 9.

Further details of oligosaccharide production and conjugation are given below.

10 ***Saccharide mixtures***

The compositions of the invention can comprise capsular saccharides from at least two (*i.e.* 2, 3 or 4) of serogroups A, C, W135 and Y of *N.meningitidis*.

- Mixtures of saccharides from more than one serogroup of *N.meningitidis* are preferred *e.g.* compositions comprising saccharides from serogroups A+C, A+W135, A+Y, C+W135, C+Y, 15 W135+Y, A+C+W135, A+C+Y, C+W135+Y, A+C+W135+Y, *etc.* It is preferred that the protective efficacy of individual saccharide antigens is not removed by combining them, although actual immunogenicity (*e.g.* ELISA titres) may be reduced.

Preferred compositions comprise saccharides from serogroups C and Y. Other preferred compositions comprise saccharides from serogroups C, W135 and Y.

- 20 Where a mixture comprises capsular saccharides from both serogroups A and C, the ratio (w/w) of MenA saccharide:MenC saccharide may be greater than 1 (*e.g.* 2:1, 3:1, 4:1, 5:1, 10:1 or higher).

- Where a mixture comprises capsular saccharides from serogroup Y and one or both of serogroups C and W135, the ratio (w/w) of MenY saccharide:MenW135 saccharide may be greater than 1 (*e.g.* 2:1, 3:1, 4:1, 5:1, 10:1 or higher) and/or that the ratio (w/w) of MenY saccharide:MenC saccharide 25 may be less than 1 (*e.g.* 1:2, 1:3, 1:4, 1:5, or lower).

Preferred ratios (w/w) for saccharides from serogroups A:C:W135:Y are: 1:1:1:1; 1:1:1:2; 2:1:1:1; 4:2:1:1; 8:4:2:1; 4:2:1:2; 8:4:1:2; 4:2:2:1; 2:2:1:1; 4:4:2:1; 2:2:1:2; 4:4:1:2; and 2:2:2:1.

Purification of capsular polysaccharides

- Meningococcal capsular polysaccharides are typically prepared by a process comprising the steps of polysaccharide precipitation (*e.g.* using a cationic detergent), ethanol fractionation, cold phenol 30 extraction (to remove protein) and ultracentrifugation (to remove LPS) [*e.g.* ref. 10].

A more preferred process [8], however, involves polysaccharide precipitation followed by solubilisation of the precipitated polysaccharide using a lower alcohol. Precipitation can be achieved using a cationic detergent such as tetrabutylammonium and cetyltrimethylammonium salts (*e.g.* the

bromide salts), or hexadimethrine bromide and myristyltrimethylammonium salts. Cetyltrimethylammonium bromide ('CTAB') is particularly preferred [11]. Solubilisation of the precipitated material can be achieved using a lower alcohol such as methanol, propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol, 2-methyl-propan-1-ol, 2-methyl-propan-2-ol, diols, *etc.*, but ethanol is particularly suitable for solubilising CTAB-polysaccharide complexes. Ethanol is preferably added to the precipitated polysaccharide to give a final ethanol concentration (based on total content of ethanol and water) of between 50% and 95%.

After re-solubilisation, the polysaccharide may be further treated to remove contaminants. This is particularly important in situations where even minor contamination is not acceptable (*e.g.* for human vaccine production). This will typically involve one or more steps of filtration *e.g.* depth filtration, filtration through activated carbon may be used, size filtration and/or ultrafiltration.

Once filtered to remove contaminants, the polysaccharide may be precipitated for further treatment and/or processing. This can be conveniently achieved by exchanging cations (*e.g.* by the addition of calcium or sodium salts).

The polysaccharide may be chemically modified. For instance, it may be modified to replace one or more hydroxyl groups with blocking groups. This is particularly useful for serogroup A [12].

Oligosaccharides

The capsular saccharides will generally be in the form of oligosaccharides. These are conveniently formed by fragmentation of purified capsular polysaccharide (*e.g.* by hydrolysis, in mild acid, or by heating), which will usually be followed by purification of the fragments of the desired size.

Fragmentation of polysaccharides is preferably performed to give a final average degree of polymerisation (DP) in the oligosaccharide of less than 30 (*e.g.* between 10 and 20, preferably around 10 for serogroup A; between 15 and 25 for serogroups W135 and Y, preferably around 15-20; between 12 and 22 for serogroup C; *etc.*). DP can conveniently be measured by ion exchange chromatography or by colorimetric assays [13].

If hydrolysis is performed, the hydrolysate will generally be sized in order to remove short-length oligosaccharides. This can be achieved in various ways, such as ultrafiltration followed by ion-exchange chromatography. Oligosaccharides with a degree of polymerisation of less than or equal to about 6 are preferably removed for serogroup A, and those less than around 4 are preferably removed for serogroups W135 and Y.

Covalent conjugation

Capsular saccharides in compositions of the invention will usually be conjugated to carrier protein(s). In general, conjugation enhances the immunogenicity of saccharides as it converts them from T-independent antigens to T-dependent antigens, thus allowing priming for immunological memory.

Conjugation is particularly useful for paediatric vaccines [e.g. ref. 14] and is a well known technique [e.g. reviewed in refs. 15 to 23, etc.].

Preferred carrier proteins are bacterial toxins or toxoids, such as diphtheria or tetanus toxoids. The CRM₁₉₇ diphtheria toxoid [24, 25, 26] is particularly preferred. Other suitable carrier proteins include the *N.meningitidis* outer membrane protein [27], synthetic peptides [28, 29], heat shock proteins [30, 31], pertussis proteins [32, 33], cytokines [34], lymphokines [34], hormones [34], growth factors [34], artificial proteins comprising multiple human CD4⁺ T cell epitopes from various pathogen-derived antigens [35], protein D from *H.influenzae* [36], toxin A or B from *C.difficile* [37], etc.

Within a composition of the invention, it is possible to use more than one carrier protein. Thus different carrier proteins can be used for different serogroups e.g. serogroup A saccharides might be conjugated to CRM₁₉₇ while serogroup C saccharides might be conjugated to tetanus toxoid. It is also possible to use more than one carrier protein for a particular saccharide antigen e.g. serogroup A saccharides might be in two groups, with some conjugated to CRM₁₉₇ and others conjugated to tetanus toxoid. In general, however, it is preferred to use the same carrier protein for all saccharides.

A single carrier protein might carry more than one saccharide antigen [38]. For example, a single carrier protein might have conjugated to it saccharides from serogroups A and C.

Conjugates with a saccharide:protein ratio (w/w) of between 0.5:1 (i.e. excess protein) and 5:1 (i.e. excess saccharide) are preferred, and those with a ratio between 1:1.25 and 1:2.5 are more preferred.

Conjugates may be used in conjunction with free carrier protein [39].

Any suitable conjugation reaction can be used, with any suitable linker where necessary.

The saccharide will typically be activated or functionalised prior to conjugation. Activation may involve, for example, cyanylating reagents such as CDAP (e.g. 1-cyano-4-dimethylamino pyridinium tetrafluoroborate [40, 41, etc.]). Other suitable techniques use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S-NHS, EDC, TSTU; see also the introduction to reference 21).

Linkages via a linker group may be made using any known procedure, for example, the procedures described in references 42 and 43. One type of linkage involves reductive amination of the polysaccharide, coupling the resulting amino group with one end of an adipic acid linker group, and then coupling a protein to the other end of the adipic acid linker group [19, 44, 45]. Other linkers include B-propionamido [46], nitrophenyl-ethylamine [47], haloacyl halides [48], glycosidic linkages [49], 6-aminocaproic acid [50], ADH [51], C₄ to C₁₂ moieties [52] etc. As an alternative to using a linker, direct linkage can be used. Direct linkages to the protein may comprise oxidation of the polysaccharide followed by reductive amination with the protein, as described in, for example, references 53 and 54.

A process involving the introduction of amino groups into the saccharide (e.g. by replacing terminal =O groups with -NH₂) followed by derivatisation with an adipic diester (e.g. adipic acid N-hydroxysuccinimido diester) and reaction with carrier protein is preferred.

After conjugation, free and conjugated saccharides can be separated. There are many suitable methods, including hydrophobic chromatography, tangential ultrafiltration, diafiltration *etc.* [see also refs. 55 & 56, *etc.*].

Where the composition of the invention includes a conjugated oligosaccharide, it is preferred that oligosaccharide preparation precedes conjugation.

Preparation of compositions of the invention

Where compositions of the invention include more than one type of capsular saccharide, they are preferably prepared separately (including any fragmentation, conjugation, *etc.*) and then admixed to give a composition of the invention.

Where the composition comprises capsular saccharide from serogroup A, however, it is preferred that the serogroup A saccharide is not combined with the other saccharide(s) until shortly before use, in order to minimise the potential for hydrolysis. This can conveniently be achieved by having the serogroup A component in lyophilised form and the other serogroup component(s) in liquid form, with the liquid component being used to reconstitute the lyophilised component when ready for use.

A composition of the invention may thus be prepared from a kit comprising: (a) capsular saccharide from *N.meningitidis* serogroup A, in lyophilised form; and (b) capsular saccharide(s) from one or more (e.g. 1, 2, 3) of *N.meningitidis* serogroups C, W135 and Y, in liquid form. The invention also provides a method for preparing a composition of the invention, comprising mixing a lyophilised capsular saccharide from *N.meningitidis* serogroup A with capsular saccharide(s) from one or more (e.g. 1, 2, 3) of *N.meningitidis* serogroups C, W135 and Y, wherein said one or more saccharides are in liquid form.

The invention also provides a composition of the invention, comprising capsular saccharide(s) from *N.meningitidis* serogroups C, W135 and Y, wherein saccharides are in liquid form. This composition may be packaged with a lyophilised serogroup A saccharide antigen, for reconstitution, or it may be used as a composition on its own e.g. where immunisation against serogroup A is not desired.

Presentation of compositions of the invention

Compositions of the invention may be presented and packaged in various ways.

Where compositions are for injection, they may be presented in vials, or they may be presented in ready-filled syringes. The syringes may be supplied with or without needles. A syringe will include a single dose of the composition, whereas a vial may include a single dose or multiple doses. Injectable compositions will usually be liquid solutions or suspensions. Alternatively, they may be presented in solid form for solution or suspension in liquid vehicles prior to injection.

Where a composition of the invention is to be prepared extemporaneously prior to use (e.g. where serogroup A saccharide is presented in lyophilised form) and is presented as a kit, the kit may comprise two vials, or it may comprise one ready-filled syringe and one vial, with the contents of the syringe being used to reactivate the contents of the vial prior to injection.

- 5 However, preferred compositions are for mucosal delivery. Of the various mucosal delivery options available, the intranasal route is the most practical as it offers easy access with relatively simple devices that have already been mass produced. The composition of the invention is thus preferably adapted for and/or packaged for intranasal administration, such as by nasal spray, nasal drops, gel or powder [e.g. refs 57 & 58].
- 10 Alternative routes for mucosal delivery of the composition are oral, intragastric, pulmonary, intestinal, transdermal, rectal, ocular, and vaginal routes. The composition of the invention may thus be adapted for and/or packaged for mucosal administration [e.g. see refs. 59, 60 & 61]. Where the composition is for oral administration, for instance, it may be in the form of tablets or capsules (optionally enteric-coated), liquid, transgenic plant material, drops, inhaler, aerosol, enteric coating,
- 15 suppository, pessary, etc. [see also ref. 62, and Chapter 17 of ref. 73].

Whatever the route of delivery, compositions of the invention are preferably packaged in unit dose form. Effective doses can be routinely established. A typical human dose of the composition for injection or for intranasal use has a volume between 0.1-0.5ml e.g. two 100µl sprays, one per nostril.

- 20 Within each dose, the amount of an individual saccharide antigen will generally be between 1-50 µg (measured as mass of saccharide), with about 10 µg of each being preferred.

Compositions of the invention are preferably sterile. They are preferably pyrogen-free. They are preferably buffered e.g. at between pH 6 and pH 8, generally around pH 7. Where a composition comprises an aluminium hydroxide salt, it is preferred to use a histidine buffer [63].

Adjuvants

- 25 The compositions will generally include one or more adjuvants. The adjuvant(s) may be added to saccharides before and/or after they are admixed to form a composition of the invention, but it is preferred to combine adjuvant with a saccharide antigen prior to admixing of different saccharides.

- However, it is not necessary that each saccharide must be adjuvanted prior to such admixing. Excess adjuvant can be included in one saccharide preparation such that, when further unadjuvanted
- 30 saccharide antigen(s) is/are added, the excess is diluted to a desired final concentration. In one particular embodiment, where the composition of the invention is prepared from a lyophilised antigen (e.g. a lyophilised serogroup A component) it may be preferred not to include an adjuvant in the lyophilised material.

- For mucosal delivery, it is preferred to use a mucosal adjuvant. Mucosal adjuvants include, but are
- 35 not limited to: (A) *E.coli* heat-labile enterotoxin ("LT"), or detoxified mutants thereof [e.g. Chapter 5

of ref. 64]; (B) cholera toxin ("CT"), or detoxified mutants thereof [e.g. Chapter 5 of ref. 64]; or (C) microparticles (*i.e.* a particle of ~100nm to ~150µm in diameter, more preferably ~200nm to ~30µm in diameter, and most preferably ~500nm to ~10µm in diameter) formed from materials that are biodegradable and non-toxic (*e.g.* a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone *etc.*, such as poly(lactide-co-glycolide) *etc.*) optionally treated to have a negatively-charged surface (*e.g.* with SDS) or a positively-charged surface (*e.g.* with a cationic detergent, such as CTAB); (D) a polyoxyethylene ether or a polyoxyethylene ester [65]; (E) a polyoxyethylene sorbitan ester surfactant in combination with an octoxynol [66] or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol [67]; (F) chitosan [e.g. 68]; (G) an immunostimulatory oligonucleotide (*e.g.* a CpG oligonucleotide) and a saponin [69]; (H) liposomes [chapters 13 & 14 of ref. 73]; (I) monophosphoryl lipid A mimics, such as aminoalkyl glucosaminide phosphate derivatives *e.g.* RC-529 [70]; (J) polyphosphazene (PCPP); (K) a bioadhesive [71] such as esterified hyaluronic acid microspheres [72] or a mucoadhesive selected from the group consisting of cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrrolidone, polysaccharides and carboxymethylcellulose. Other mucosal adjuvants are also available [e.g. see chapter 7 of ref. 73].

In addition to the mucosal adjuvants given above, the compositions of the invention may include one or more further adjuvants selected from the following group: (A) aluminium salts (alum), such as aluminium hydroxides (including oxyhydroxides), aluminium phosphates (including hydroxyphosphates), aluminium sulfate, *etc.* [Chapters 8 & 9 in ref. 73]; (B) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides [Muramyl peptides include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE], *etc.*] or bacterial cell wall components), such as for example (a) MF59™ [Chapter 10 in ref. 73; 74, 75], containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing MTP-PE) formulated into submicron particles using a microfluidizer, (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (C) saponin adjuvants [chapter 22 of ref. 73], such as QS21 or Stimulon™ (Cambridge Bioscience, Worcester, MA), either in simple form or in the form of particles generated therefrom such as ISCOMs (immunostimulating complexes; chapter 23 of ref. 73), which ISCOMS may be devoid of additional detergent *e.g.* ref. 76; (D) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (E) cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 [77], *etc.*), interferons (*e.g.* gamma interferon), macrophage colony stimulating factor (M-CSF),

- tumor necrosis factor (TNF), *etc.*; (F) monophosphoryl lipid A (MPL) or 3-O-deacylated MPL (3dMPL) *e.g.* refs. 78 & 79, optionally in the substantial absence of alum when used with pneumococcal saccharides *e.g.* ref. 80; (G) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions *e.g.* refs. 81, 82 & 83; (H) oligonucleotides comprising CpG motifs *i.e.* containing at least one CG dinucleotide, with 5-methylcytosine optionally being used in place of cytosine; (I) an immunostimulant and a particle of metal salt *e.g.* ref. 84; (J) a saponin and an oil-in-water emulsion *e.g.* ref. 85; (K) a saponin (*e.g.* QS21) + 3dMPL + IL-12 (optionally + a sterol) *e.g.* ref. 86; (L) double-stranded RNA; (M) other substances that act as immunostimulating agents to enhance the effectiveness of the composition [*e.g.* chapter 7 of ref. 73].
- Where an aluminium phosphate is used, it is possible to adsorb one or more of the saccharides to the aluminium salt, but it is preferred not to do so, and this is favoured by including free phosphate ions in solution (*e.g.* by the use of a phosphate buffer). Where an aluminium hydroxide is used, it is preferred to adsorb the saccharides to the salt. The use of aluminium hydroxide as an adjuvant may be preferred for saccharide from serogroup A.
- Preferred mucosal adjuvants are chitosan (including trimethylchitosan) and detoxified mutants of bacterial toxins (particularly LT.) These can be used alone, or can advantageously be used in combination, as co-administration allows lower doses of the toxin to be used, thereby improving safety. Moreover, whereas chitosan alone gives a Th2-biased response, the addition of LTK63 can cause a shift towards a Th1-biased response.
- Chitosan**
- Chitosan is known for use as an adjuvant [*e.g.* refs. 87 to 98], particularly for mucosal (*e.g.* intranasal) use. Chitosan (Figure 11) is a N-deacetylated derivative of the exoskeletal polymer chitin (Figure 12), although the N-deacetylation is almost never complete. The deacetylation means that, unlike chitin, chitosan is soluble in dilute aqueous acetic and formic acids. Chitosan has also found wide applicability in non-vaccine pharmaceutical fields [99].
- The repeating glucosamine monomer of chitosan contains an amine group. This group may exist as free amine ($-\text{NH}_2$) or as cationic amine ($-\text{NH}_3^+$), with protonation affecting the polymer's solubility. The amine groups are chemically active and can be substituted. Of particular interest for the invention, the amine groups can be substituted with one or more alkyl group ('A' *e.g.* methyl, ethyl, propyl, butyl, pentyl, *etc.*) *e.g.* $-\text{NHA}$, $-\text{NH}_2\text{A}^+$, $-\text{NA}^1\text{A}^2$, $-\text{NHA}^1\text{A}^2$, $-\text{NA}^1\text{A}^2\text{A}^3$. Preferred derivatives are tri-alkylated and particularly preferred derivatives are trimethylated (*i.e.* trimethylchitosan, or 'TMC' — Figure 13). These derivatives have much higher aqueous solubility than unmodified chitosan over a broader pH range.
- It is not necessary for every amine in the chitosan polymer to be substituted in this way. The degree of substitution along the length of the chitosan chain can be determined by $^1\text{H-NMR}$ and can be controlled by means of the number and duration of reaction steps [100]. It is preferred that at least

10% (e.g. at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more) of monomers have a substituted amine.

There are 2 main reasons why it is rare that 100% of monomers in the chitosan will carry an alkylated amine. First, the substitution reaction will not usually be 100% efficient. Second, it is rare to find
 5 chitosan in which 100% of the monomer units carry amine groups because deacetylation of chitin is not usually 100% efficient. Alkylated chitosan derivatives used in the invention may therefore have amide and/or non-alkylated groups on some monomer units, and chitosan may possess some amide groups. Chitosan and derivatives used with the invention are preferably at least 75% deacetylated.

Chitosans come in a variety of molecular weights e.g. from oligosaccharides with molecular weight
 10 around 5,000-10,000 to polymers of high molecular weight (e.g. 600,000 – 1,000,000).

Where a cationic chitosan or derivative is used, it will be in the form of a salt e.g. chloride or lactate.

The chitosan or derivative can take various physical forms e.g. in solution, as a powder, or in particulate form. Particulate forms are preferred, including microparticles, which may be cross-linked or non-cross-linked and may be formed conveniently by spray-drying [101, 102]. Other physical
 15 forms include gels, beads, films, sponges, fibres, emulsions, etc.

The term “chitosan” as used with reference to the compositions, processes, methods and uses of the invention includes all these forms and derivatives of chitosan.

Detoxified mutant toxins

ADP-ribosylating bacterial exotoxins which catalyse the transfer of an ADP-ribose unit from NAD⁺
 20 to a target protein are widely known. Examples include diphtheria toxin (*Corynebacterium diphtheriae*), exotoxin A (*Pseudomonas aeruginosa*), cholera toxin (CT; *Vibrio cholerae*), heat-labile enterotoxin (LT; *E.coli*) and pertussis toxin (PT). Further examples are in references 103 & 104.

The toxins are typically divided into two functionally distinct domains – A and B. The A subunit is responsible for the toxic enzymatic activity, whereas the B subunit is responsible for cellular binding.
 25 The subunits might be domains on the same polypeptide chain, or might be separate polypeptide chains. The subunits may themselves be oligomers e.g. the A subunit of CT consists of A₁ and A₂ which are linked by a disulphide bond, and its B subunit is a homopentamer. Typically, initial contact with a target cell is mediated by the B subunit and then subunit A alone enters the cell.

The toxins are typically immunogenic, but their inclusion in vaccines is hampered by their toxicity.
 30 To remove toxicity without also removing immunogenicity, the toxins have been treated with chemicals such as glutaraldehyde or formaldehyde. A more rational approach relies on site-directed mutagenesis of key active site residues to remove toxic enzymatic activity whilst retaining immunogenicity [e.g. refs. 105 (CT and LT), 106 (PT), 64 etc.]. Current acellular whooping cough vaccines include a form of pertussis toxin with two amino acid substitutions (Arg⁹→Lys and
 35 Glu¹²⁹→Gly; ‘PT-9K/129G’ [107]).

As well as their immunogenic properties, the toxins have been used as adjuvants. Parenteral adjuvanticity was first observed in 1972 [108] and mucosal adjuvanticity in 1984 [109]. It was surprisingly found in 1993 that the detoxified forms of the toxins retain adjuvanticity [110].

5 The compositions of the invention include a detoxified ADP-ribosylating toxin. The toxin may be diphtheria toxin, *Pseudomonas* exotoxin A or pertussis toxin, but is preferably cholera toxin (CT) or, more preferably, *E.coli* heat-labile enterotoxin (LT). Other toxins which can be used are those disclosed in reference 104 (SEQ IDs 1 to 7 therein, and mutants thereof).

10 Detoxification of these toxins without loss of immunogenic and/or adjuvant activity can be achieved by any suitable means, with mutagenesis being preferred. Mutagenesis may involve one or more substitutions, deletions and/or insertions.

Preferred detoxified mutants are LT having a mutation at residue Arg-7 (*e.g.* a Lys substitution); CT having a mutation at residue Arg-7 (*e.g.* a Lys substitution); CT having a mutation at residue Arg-11 (*e.g.* a Lys substitution); LT having a mutation at Val-53; CT having a mutation at Val-53; CT having a mutation at residue Ser-61 (*e.g.* a Phe substitution); LT having a mutation at residue Ser-63
15 (*e.g.* a Lys or Tyr substitution) [*e.g.* Chapter 5 of ref. 64 – K63; ref. 111 – Y63]; CT having a mutation at residue Ser-63 (*e.g.* a Lys or Tyr substitution); LT having a mutation at residue Ala-72 (*e.g.* an Arg substitution) [112 – R72]; LT having a mutation at Val-97; CT having a mutation at Val-97; LT having a mutation at Tyr-104; CT having a mutation at Tyr-104; LT having a mutation at residue Pro-106 (*e.g.* a Ser substitution); CT having a mutation at residue Pro-106 (*e.g.* a Ser
20 substitution); LT having a mutation at Glu-112 (*e.g.* a Lys substitution); CT having a mutation at Glu-112 (*e.g.* a Lys substitution); LT having a mutation at residue Arg-192 (*e.g.* a Gly substitution); PT having a mutation at residue Arg-9 (*e.g.* a Lys substitution); PT having a mutation at Glu-129 (*e.g.* a Gly substitution); and any of the mutants disclosed in reference 105.

25 These mutations may be combined *e.g.* Arg-9-Lys + Glu-129-Gly in PT, or LT with both a D53 and a K63 mutation, *etc.*

LT with a mutation at residue 63 or 72 is a preferred detoxified toxin. The LT-K63 and LT-R72 toxins are particularly preferred [113].

It will be appreciated that the numbering of these residues is based on prototype sequences and that, for example, although Ser-63 may not actually be the 63rd amino acid in a given LT variant, an
30 alignment of amino acid sequences will reveal the location corresponding to Ser-63.

The detoxified toxins may be in the form of A and/or B subunits as appropriate for adjuvant activity.

Further components of the compositions

In addition to meningococcal saccharide antigens, compositions of the invention may include meningococcal protein antigens. It is preferred to include proteins from serogroup B of
35 *N.meningitidis* [*e.g.* refs. 114 to 119] or OMV preparations [*e.g.* refs. 120 to 123 *etc.*].

Non-meningococcal and non-neisserial antigens, preferably ones that do not diminish the immune response against the meningococcal components, may also be included. Ref. 124, for instance, discloses combinations of oligosaccharides from *N.meningitidis* serogroups B and C together with the Hib saccharide. Antigens from pneumococcus, hepatitis A virus, hepatitis B virus, *B.pertussis*,
 5 diphtheria, tetanus, *Helicobacter pylori*, polio and/or *H.influenzae* are preferred. Particularly preferred non-neisserial antigens include:

- antigens from *Helicobacter pylori* such as CagA [125 to 128], VacA [129, 130], NAP [131, 132, 133], HopX [e.g. 134], HopY [e.g. 134] and/or urease.
- a saccharide or protein antigen from *Streptococcus pneumoniae* [e.g. 135, 136, 137].
- 10 - an antigen from hepatitis A virus, such as inactivated virus [e.g. 138, 139].
- an antigen from hepatitis B virus, such as the surface and/or core antigens [e.g. 139, 140], with surface antigen preferably being adsorbed onto an aluminium phosphate [141].
- a saccharide antigen from *Haemophilus influenzae* B [e.g. 9], preferably non-adsorbed or adsorbed onto an aluminium phosphate [142].
- 15 - an antigen from hepatitis C virus [e.g. 143].
- an antigen from *N.gonorrhoeae* [e.g. 114 to 117].
- an antigen from *Chlamydia pneumoniae* [e.g. refs. 144 to 145, 146, 147, 148, 149, 150].
- an antigen from *Chlamydia trachomatis* [e.g. 151].
- an antigen from *Porphyromonas gingivalis* [e.g. 152].
- 20 - polio antigen(s) [e.g. 153, 154] such as IPV.
- rabies antigen(s) [e.g. 155] such as lyophilised inactivated virus [e.g. 156, RabAvert™].
- measles, mumps and/or rubella antigens [e.g. chapters 12, 13 & 17 of ref. 1].
- influenza antigen(s) [e.g. chapter 21 of ref.1], such as the haemagglutinin and/or neuraminidase surface proteins.
- 25 - an antigen from *Moraxella catarrhalis* [e.g. 157].
- an antigen from *Streptococcus agalactiae* (group B streptococcus) [e.g. 158, 159].
- an antigen from *Streptococcus pyogenes* (group A streptococcus) [e.g. 159, 160, 161].
- an antigen from *Staphylococcus aureus* [e.g. 162].
- antigen(s) from a paramyxovirus such as respiratory syncytial virus (RSV [163, 164]) and/or
 30 parainfluenza virus (PIV3 [165]).
- an antigen from *Bacillus anthracis* [e.g. 166, 167, 168].
- an antigen from a virus in the flaviviridae family (genus flavivirus), such as from yellow fever virus, Japanese encephalitis virus, four serotypes of Dengue viruses, tick-borne encephalitis virus, West Nile virus.
- 35 - a pestivirus antigen, such as from classical porcine fever virus, bovine viral diarrhoea virus, and/or border disease virus.
- a parvovirus antigen e.g. from parvovirus B19.
- a tetanus toxoid [e.g. chapter 18 of ref. 1]

- pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B.pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3 [e.g. refs. 169 & 170].
- cellular pertussis antigen.

The mixture may comprise one or more of these further antigens, which may be detoxified where necessary (e.g. detoxification of pertussis toxin by chemical and/or genetic means).

Where a diphtheria antigen is included in the mixture it is preferred also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus antigen is included it is preferred also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is preferred also to include diphtheria and tetanus antigens.

Antigens in the mixture will typically be present at a concentration of at least 1µg/ml each. In general, the concentration of any given antigen will be sufficient to elicit an immune response against that antigen.

It may be preferred not to include all three of (1) a meningococcal saccharide, (2) an antigen which induces an immune response against *Haemophilus influenzae*, and (3) an antigen which induces an immune response against *Streptococcus pneumoniae* together in the composition of the invention. If these three antigens are included in the same composition, however, it is preferred that the composition includes an alkylated derivative of chitosan (e.g. trimethylchitosan) as an adjuvant.

As an alternative to using proteins antigens in the mixture, nucleic acid encoding the antigen may be used. Protein components of the mixture may thus be replaced by nucleic acid (preferably DNA e.g. in the form of a plasmid) that encodes the protein. Similarly, compositions of the invention may comprise proteins which mimic saccharide antigens e.g. mimotopes [171] or anti-idiotypic antibodies. These may replace individual saccharine components, or may supplement them. As an example, the vaccine may comprise a peptide mimic of the MenC [172] or the MenA [173] capsular polysaccharide in place of the saccharide itself.

Compositions of the invention may comprise detergent (e.g. a Tween, such as Tween 80) at low levels (e.g. <0.01%). Compositions of the invention may comprise a sugar alcohol (e.g. mannitol) or trehalose e.g. at around 15mg/ml, particularly if they are to be lyophilised or if they include material which has been reconstituted from lyophilised material.

Immunogenicity

Compositions of the invention are immunogenic. Preferred immunogenic compositions are vaccines. Vaccines according to the invention may either be prophylactic (i.e. to prevent infection) or therapeutic (i.e. to treat disease after infection), but will typically be prophylactic.

Immunogenic compositions and vaccines of the invention will, in addition to the meningococcal saccharides, typically comprise 'pharmaceutically acceptable carriers', which include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolised macromolecules such as pro-

teins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, trehalose [174], lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. The vaccines may also contain diluents, such as water, saline, glycerol, *etc.* Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present. A thorough discussion of pharmaceutically acceptable excipients is available in ref. 175.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of saccharide antigen, as well as any other of the above-mentioned components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (*e.g.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Immunogenicity of compositions of the invention can be determined by administering them to test subjects (*e.g.* children 12-16 months age, or animal models [176]) and then determining standard parameters including serum bactericidal antibodies (SBA) and ELISA titres (GMT) of total and high-avidity anti-capsule IgG. These immune responses will generally be determined around 4 weeks after administration of the composition, and compared to values determined before administration of the composition. A SBA increase of at least 4-fold or 8-fold is preferred. Where more than one dose of the composition is administered, more than one post-administration determination may be made.

Administration of compositions of the invention

As mentioned above, compositions of the invention may be administered by various routes, including parenteral and mucosal. A preferred route of parenteral administration is injection. Injection may be subcutaneous, intraperitoneal, intravenous or intramuscular. Intramuscular administration to the thigh is preferred. Needle-free injection may be used. A preferred route of mucosal administration is intranasal. Transdermal or transcutaneous administration is also possible (*e.g.* see ref. 177).

Administration may be a single dose schedule or a multiple dose schedule. A primary dose schedule may be followed by a booster dose schedule. Suitable timing between priming and boosting can be routinely determined.

Administration will generally be to an animal and, in particular, human subjects can be treated. The compositions are particularly useful for vaccinating children and teenagers.

Medical methods and uses

The invention provides a method of raising an immune response in a patient, comprising administering to the patient a composition of the invention. The immune response is preferably protective against meningococcal disease, and may comprise a humoral immune response and/or a
 5 cellular immune response. The immune response and/or the administration is/are preferably both mucosal.

The patient is preferably a child. A further preferred class of patient is an adult woman, and particularly a woman of child-bearing age or a pregnant woman. Compositions of the invention are particularly suited for passively immunising children via the maternal route.

10 The method may raise a booster response, in a patient that has already been primed against *N.meningitidis*.

The invention also provides the use of capsular saccharides from at least two of serogroups A, C, W135 and Y of *N.meningitidis*, wherein said capsular saccharides are conjugated to carrier protein(s) and/or are oligosaccharides, in the manufacture of a medicament for intranasal delivery to an animal in
 15 order to raise an immune response. The invention also provides the use of (1) a capsular saccharide from at least one of serogroups A, C, W135 and Y of *N.meningitidis*, wherein said capsular saccharides are conjugated to carrier protein(s) and/or are oligosaccharides, and (2) a chitosan, in the manufacture of a medicament for intranasal delivery to an animal in order to raise an immune response. The use may also involve (3) a detoxified ADP-ribosylating toxin.

20 These medicaments are preferably for the prevention and/or treatment of a disease caused by a *Neisseria* (e.g. meningitis, septicaemia, gonorrhoea etc.). They are preferably for intranasal administration. They preferably comprise capsular saccharides from at least two (i.e. 2, 3 or 4) of serogroups A, C, W135 and Y of *N.meningitidis*.

Th1/Th2 bias

25 Vaccines compositions comprising chitosan (including derivates thereof) and antigens are known in the art. Chitosan gives a Th2-biased immune response. It has been found that the addition of detoxified ADP-ribosylating toxin adjuvants (e.g. LT mutants, such as LTK63) to these vaccines can shift the immune response to have a Th1-bias. The invention thus provides a vaccine composition comprising a chitosan adjuvant, a mutant ADP-ribosylating toxin and an antigen, wherein the
 30 vaccine composition gives a Th1-biased immune response after administration to a subject. The invention also provides a method for altering the Th1/Th2 balance of a chitosan-containing vaccine, comprising the step of adding a detoxified ADP-ribosylating toxin to the vaccine.

Definitions

The term "comprising" means "including" as well as "consisting" e.g. a composition "comprising" X
 35 may consist exclusively of X or may include something additional e.g. X + Y.

The term "about" in relation to a numerical value x means, for example, $x \pm 10\%$.

The word "substantially" does not exclude "completely" e.g. a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

BRIEF DESCRIPTION OF DRAWINGS

5 Figure 1 illustrates the preparation of an oligosaccharide conjugate.

Figures 2, 5 & 8 shows serum IgG data from the examples. Figures 3, 6 & 9 shows serum BCA data from the examples. Figures 4, 7 & 10 shows spleen proliferation data from the examples.

Figures 11 to 13 show the repeating structures of (11) chitosan (12) chitin and (13) trimethylchitosan.

Figure 14 shows IgG ELISA titres (14A) and bactericidal titres (14B) using TMC and/or LT-K63.

10 Figure 15 shows IgA titres in serum (15A) and nasal washes (15B) for the same experiments, and Figure 16 shows results of a spleen proliferation assay varying with CRM₁₉₇ concentration ($\mu\text{g/ml}$).

Figure 17 shows serum IgG titres obtained after three doses of MenC antigen with chitosan adjuvant. Figure 18 shows nasal IgA titres for the same experiments, and Figure 19 shows serum bactericidal antibodies for the same experiments.

15 Figures 20 and 21 show serum anti-MenC IgG antibody titres (top; mean titre \pm SD) measured by ELISA after 1 (post-1), after 2 (post-2), and after 3 immunizations (post-3) and serum bactericidal antibody titres (bottom) tested on pooled samples obtained after three immunizations.

Figure 22 shows MenC-specific IgG1, IgG2a, and IgE antibody titers by ELISA after 3 immunizations. Each value represents the mean titer \pm SD.

20 Figures 23 and 24 show IL-5 and IFN- γ responses using (23) TMC or (24) LTK63.

MODES FOR CARRYING OUT THE INVENTION

Meningococcal serogroup C vaccine [182]

A CRM₁₉₇ meningococcal C oligosaccharide conjugate [6,9] was administered intranasally at $1\mu\text{g}$ per dose (measured as saccharide) to mice using N-trimethyl-chitosan chloride [178] and/or LT-K63
 25 adjuvants. TMC was used as $8\mu\text{g}$ per dose, and was prepared [179] from chitosan ('Chitoclear', Primex ehf, Iceland) from shrimp shells (94.5% acetylated) with 18.9% substitution. LT-K63 was used at 1 or $0.1\mu\text{g}$ per dose. Unanesthetized female BALB/c were immunized intranasally on days 0, 21, 35 with the formulations in $10\mu\text{l}$ volumes ($5\mu\text{l}$ per nostril). Serum samples were taken before and after each immunization. Nasal washes were taken ten days after the third immunization. IgG and
 30 IgA antibody titers specific for MenC and for LT were determined by ELISA [180]. Control mice received a $400\mu\text{l}$ volume subcutaneously (s.c.), including $500\mu\text{g}$ of aluminium hydroxide adjuvant. All formulations were prepared in PBS pH 7.4 just before use by mixing the CRM-MenC conjugate vaccine with the adjuvant for s.c. immunisations, or with a powder suspension of TMC, plus or minus the LTK63 mucosal adjuvant.

Serum samples were taken on days 0, 20 (post-1), 34 (post-2), and 45 (post-3), when mice were sacrificed, nasal washes were taken and spleens were removed. Nasal washes were performed by repeated flushing and aspiration of 1 ml of PBS, pH 7.4 containing 0.1% bovine serum albumin (BSA) and 1 mM phenylmethanesulfonyl fluoride (PMSF).

- 5 Titration of serum and mucosal anti-MenC-, anti-CRM₁₉₇- and anti-LTK63-specific IgG and IgA antibodies was carried out by ELISA on individual serum samples as previously detailed [180-182]. Antibody titres were statistically compared with a two-tailed Student's test. Serum bactericidal activity against *N.meningitidis* groups C strain C11 was titred on pooled serum samples according to the standard procedures already described [119,180] using baby rabbit serum as a source of
10 complement.

Serum IgG responses are shown in Figure 14: (A) ELISA and (B) bactericidal (log scale). Figure 15 shows IgA titres in (A) serum and (B) nasal washes. Figure 16 shows the results of a spleen proliferation assay.

- The data show that TMC alone enhances immunogenicity and also that TMC enhances
15 immunogenicity when co-administered with LT-K63 adjuvant. The mice receiving 1µg LT-K63 and TMC combined achieved IgG titres comparable to those obtained by subcutaneous immunisation. Moreover, the combined adjuvants at both doses gave equal or better serum bactericidal antibody responses than subcutaneous immunisation. Subcutaneous immunisation did not give rise to a MenC-specific IgA response in nasal washes.

- 20 TMC and LTK-63 are thus effective intranasal adjuvants for MenC saccharide antigen, either alone or in combination. Advantageously, the addition of TMC to LT-K63 allows the dose of LT-K63 to be reduced by 90% without loss of immunogenicity. TMC thus allows components with potential residual toxicity to be reduced without loss of immunogenicity.

In further experiments, the following nine compositions were compared:

Group	1	2	3	4	5	6	7	8	9
Alum	+	-	-	-	-	-	-	-	-
LT-K63	-	-	1µg	-	-	-	1µg	1µg	1µg
TMC	-	-	-	10µg	20µg	50µg	10µg	20µg	50µg
Route	s.c.	i.n.	i.n.	i.n.	i.n.	i.n.	i.n.	i.n.	i.n.

- 25 As shown in Figure 20, these results confirm that the highest serum anti-MenC IgG antibody titres were obtained in groups of mice which had been immunized i.n. with CRM-MenC vaccine together with both the LTK63 mutant and chitosan or TMC (groups 7 to 9). Antibody titres were comparable ($P > 0.05$) to those found in mice immunized with the same dose of vaccine given s.c. (group 1) except for the response after the first immunization which induced detectable antibodies only in s.c.
30 immunized mice. Increasing the dose of TMC from 10µg to 50µg induced a significant enhancement of the serum anti-MenC antibody response ($P < 0.01$ for TMC 10µg [group 4] versus TMC 50µg

- [group 6]), to levels comparable to those observed in mice immunized i.n. with the vaccine with 1 µg of the LTK63 mutant alone (group 3). Significant enhancement of the anti-MenC antibody response by addition of 1µg LTK63 mutant to the vaccine formulations was evident in groups of mice receiving the lowest dose of TMC (10µg) ($P < 0.01$ for group 7 versus group 4). Only mice immunized i.n. with the CRM-MenC vaccine plus adjuvants, but not those immunized s.c., had detectable serum IgA antibodies against MenC, irrespective of the adjuvant / TMC dose utilized. Finally, the concomitant use of both the LTK63 mucosal adjuvant and TMC induced bactericidal titres (1:16,000) much higher than those induced by LTK63 alone (1:4,000), by TMC alone (from 1:1,000 to 1:4,000), and by the vaccine given s.c. (1:4,000).
- 10 Similar experiments were performed using unmethyated 'Chitoclear' chitosan as adjuvant. Mice received the same conjugate antigen at 2.5µg saccharide per dose, but with LT-K63 (1µg) and/or chitosan (10 or 20µg), by the same route. Six groups of mice were used:

Group	1	2	3	4	5	6
Alum	+	—	—	—	—	—
LT-K63	—	+	—	—	+	+
Chitosan	—	—	10µg	20µg	10µg	20µg
Route	s.c.	i.n.	i.n.	i.n.	i.n.	i.n.

- As shown in Figures 17 to 19, intranasal administration with LT-K63 and chitosan, in comparison to subcutaneous administration with alum, gave equivalent IgG and serum bactericidal responses, and resulted in nasal IgA responses.

LKK63 dose reduction

To assess whether the use of TMC as co-adjuvant would allow a reduction in dose of LTK63 without loss of overall mucosal adjuvanticity, the following compositions were tested:

Group	1	2	3	4	5	6	7	8	9
Alum	+	—	—	—	—	—	—	—	—
LT-K63	—	—	—	0.05µg	0.1µg	1µg	0.05µg	0.1µg	1µg
TMC	—	—	10µg	—	—	—	10µg	10µg	10µg
Route	s.c.	i.n.	i.n.	i.n.	i.n.	i.n.	i.n.	i.n.	i.n.

- As shown in Figure 21, the strong adjuvanticity of LTK63 at 1µg per dose (group 6) dramatically drops when the mutant is used at dosages of 0.1 or 0.05 µg (groups 4 and 5). The concomitant use of TMC together with the LTK63 mutant fully restored the serum anti-MenC antibody responses (groups 7 to 9).

- Bactericidal antibody responses were negligible in mice immunised with limiting doses of the LTK63 mutant (groups 4 and 5). When LTK63 was co-administered with TMC (groups 7 to 9), however, bactericidal antibody titres increased at levels comparable to or higher than those found in mice that had received the CRM-MenC conjugate vaccine s.c. (group 1). Mice immunized i.n. with

the CRM-MenC vaccine plus LTK63 with or without TMC, but not those immunized s.c., had detectable IgA antibodies against MenC in the nasal washes.

- Thus the additive effect of TMC and of the LTK63 mutant is very well exerted at limiting doses of each other, so that the use of full doses of the LTK63 mutant would reduce the requirements for the TMC, and the use of full doses of TMC would limit the amounts of the LTK63 mutant necessary for induction of strong and protective antibody responses against MenC. Indeed, at very low doses of the LTK63 adjuvant (*i.e.* 0.1 or 0.05 μ g) high bactericidal antibody titres were induced if the CRM-MenC conjugate vaccine was co-administered together with TMC, but not in its absence. This was also true for enhancement of the immune response to the CRM carrier and to the LTK63 itself (not shown).
- These data clearly show that the intrinsic mucosal adjuvanticity of these molecules can be efficiently enhanced by formulation together with appropriate bioadhesive materials. It is, thus, expected that the safety profile of i.n. delivered vaccines would be further enhanced by concomitant use of the non-toxic LT mutant and of the TMC. The data show that protective immune responses to meningococcal conjugate vaccines can be improved by mucosal immunization using the association of two appropriate mucosal adjuvants. In particular, the quality of this protective immune response can be modulated, depending on the appropriate dosing of the mucosal adjuvants.

Th1 / Th2 bias

- It is known that i.n. immunization using CRM₁₉₇ formulated with chitosan drives the immune response preferentially towards a functional Th2-type phenotype [183,184], whereas LT mutants, and the non-toxic LTK63 mutant in particular, preferentially polarize the antigen-specific immune response after i.n. immunization towards a Th1/Th0 functional phenotype [185-187]. The Th1/Th2 balance of compositions of the invention was studied, and the use of LTK63 or TMC adjuvants was found to finely modulate the propensity of these two components to induce Th1- or Th2-type responses depending on the doses used.

- Mice were immunised as described above. Groups received the following:

Group	1	2	3	4	5	6	7	8	9
Alum	+	-	-	-	-	-	-	-	-
LT-K63	-	-	-	0.05 μ g	0.1 μ g	1 μ g	0.05 μ g	0.1 μ g	1 μ g
TMC	-	-	10 μ g	-	-	-	10 μ g	10 μ g	10 μ g
Route	s.c.	i.n.	i.n.	i.n.	i.n.	i.n.	i.n.	i.n.	i.n.

- Spleens from individual mice were dissected, cells from each group of mice were pooled together and resuspended in DMEM containing 10% fetal bovine serum, 2mM L-glutamine, 25mM Hepes, 100U penicillin and streptomycin, and 5mM 2-mercapto-ethanol. 2×10^5 cells were seeded in 200 μ l cultures in U-bottomed 96-well plates and stimulated for 5 days with CRM-MenC conjugate at different concentrations, as indicated. Cell proliferation was determined by addition of 1 μ Ci of 3 [H]-

thymidine per well 16 hours before ending the culture. Cells were then harvested onto filter paper, and incorporated radioactivity was measured in a scintillation counter.

Supernatants from triplicate cell cultures stimulated with the highest concentration of antigen were pooled and tested by ELISA to evaluate the levels of IFN- γ and of IL-5 using rat anti-murine cytokine-specific monoclonal antibodies. Briefly, 96-well plates were coated with appropriate amounts of anti-mouse IFN- γ or IL-5 antibodies diluted in 0.1 M bicarbonate buffer. After overnight incubation at 4°C, washing, and saturation of uncoated sites with 1% BSA for 2 hours at room temperature, supernatants were added to the wells and incubated overnight at 4°C. Bound cytokines were determined using biotinylated anti-IFN- γ or anti-IL-5 antibodies followed by addition of horseradish peroxidase-labeled streptavidin for 1 hour at 37°C. Bound antibodies were revealed with the o-phenylenediamine substrate followed by reading of the plates at 450 nm using a microplate ELISA reader. Cytokine concentrations were determined through the generation of a standard curve made with known amounts of recombinant murine IFN- γ or IL-5.

As shown in Figure 22, i.n. immunization with the CRM-MenC vaccine plus the LTK63 alone induced anti-MenC IgG antibodies of both IgG1 and IgG2a isotypes when it was given at the highest dose (1 μ g, group 6). However, at lower doses (*i.e.* 0.1 and 0.05 μ g) anti-MenC IgG1 were detectable, although at low titres, while IgG2a were undetectable. With TMC alone, only anti-MenC IgG1 antibodies were detectable. The concomitant administration of the LTK63 mutant and of TMC with the CRM-MenC vaccine not only enhanced the titres of anti-MenC IgG1 ($p < 0.05$ for groups 7, 8, and 9 vs. group 3) antibodies, but also induced IgG2a antibodies, especially at lower doses of LTK63 (groups 7 and 8). As expected, s.c. immunization in the presence of aluminium hydroxide induced higher titres of IgG1 to MenC as compared to IgG2a, and importantly also of IgE, which were never detectable in mice receiving the vaccine i.n. All these data strongly confirm the propensity of the LTK63 to induce Th1-dependent antigen-specific IgG isotypes (IgG2a), even in the presence of compounds like TMC able to prime Th2 responses.

Intranasal immunisation with the CRM-MenC vaccine in the presence of LTK63 or of TMC induced a priming of T-cells that specifically proliferated upon *in vitro* re-stimulation with antigen. In addition, the proliferative response was enhanced when mice had received the vaccine with both LTK63 and TMC at levels similar or higher than those observed in mice receiving the conjugate vaccine s.c.

As shown in Figure 23, i.n. immunization with the vaccine plus TMC alone induced production of both IL-5 and IFN- γ . The increase of the amount of TMC used for immunization strongly suppressed the amount of IFN- γ , but not that of IL-5 produced. Addition of the LTK63 mutant (1 μ g per dose) to the vaccine formulation with TMC dramatically suppressed the ability of cells to produce IL-5 and at the same time induced high levels of IFN- γ , similar to those detectable in culture supernatants from mice immunized with the vaccine plus LTK63 alone. The ability of LTK63 to induce production of IFN- γ was significantly reduced when used at lower doses (0.1 or 0.05 μ g) for i.n. immunization

(Figure 24). Addition of TMC to the vaccine formulation did not change the pattern of IFN- γ production at the highest dose of LTK63 (1 μ g); conversely, it favoured the production of IL-5 when the LTK63 was given at lower doses. Taken together, these data show (i) the propensity of the LTK63 to polarize the immune response towards a functional Th1-type phenotype (mainly at higher doses), (ii) the propensity of TMC to favor a functional Th2-type immune response, (iii) the possibility to modulate the balance between Th1- and Th2-type responses with an appropriate dosage and "blending" of the two components.

Previous studies of Th1/Th2 balance for chitosan and LTK36 have used fixed, high doses of LTK63 (1 μ g or higher) or of chitosan. Using decreasing doses of both components, however, the Th2-type and the Th1-type responses primed by TMC and by LTK63 can be seen more easily. At the lowest doses of TMC and of LTK63, the polarization of the immune response was much less evident. When 1 μ g of LTK63 was added to the vaccine formulations containing any doses of TMC, the immune response was consistently biased towards a Th1-type response, with production of IFN- γ and with total suppression of IL-5 production. These data strongly suggest the preeminent Th1-inducing role of LTK63 to overwhelm the Th2-biased (IL-5 producing) response of TMC. Indeed, production of IL-5 was maintained only in those groups which had received the highest doses of TMC together with the lowest doses of LTK63. The inclusion of a LTK63 mutant favours a Th1-type immune response that otherwise would have been driven towards a Th2 functional phenotype by i.n. TMC used alone or by s.c. alum.

Taken together, the data show that polarisation of immune response towards a preferential Th1 or Th2 functional phenotype is not only driven by the particular mucosal adjuvant present in the vaccine formulation, but importantly also by the relative amount of each of the components present in the formulation of the i.n. delivered vaccine. Thus, the quality of this protective immune response can be finely tailored, depending on the effector functions required for protection, by appropriate dosing of the mucosal adjuvants and delivery systems.

Combined vaccine

A combined ACWY composition of oligosaccharide conjugates was prepared using the materials described in reference 8. The composition was buffered at pH 7.4 with PBS. The concentration of each conjugate was:

	Saccharide concentration (μ g/ml)	CRM ₁₉₇ concentration (μ g/ml)
A	487.50	1073.4
C	656.00	968.5
W	939.70	918.0
Y	583.70	837.1

The composition was administered intranasally to mice in 10 μ l volumes (5 μ l per nostril) without adjuvant or with one of the following mucosal adjuvants:

Adjuvant	Concentration ($\mu\text{g}/\text{dose}$)
LT-K63	1
Chitosan	25
Trimethylchitosan (TMC)	25
LT-K63 + TMC	As above (1 + 25)

For comparison, the same antigen composition was administered subcutaneously with an aluminium hydroxide adjuvant.

As a control, the MenC conjugate alone was administered with the same adjuvants by the same routes at an equivalent concentration as the MenC in the combination composition.

- 5 Ten groups of mice therefore received the following compositions:

#	Antigen	Antigen (μg)	Adjuvant	Adjuvant (μg)
1	ACWY	4	Alum (s.c.)	500
2	C	1	Alum (s.c.)	500
3	ACWY	4	—	—
4	C	1	—	—
5	ACWY	4	LTK63	1
6	C	1	LTK63	1
7	ACWY	4	TMC	25
8	C	1	TMC	25
9	ACWY	4	TMC+LTK63	25 + 1
10	C	1	TMC+LTK63	25 + 1

In a first set of experiments, serum IgG levels following 3 intranasal doses (subcutaneous for alum) were as follows, expressed as GMT (MEU/ml) \pm standard deviation (Figure 2):

#	Anti-MenA	Anti-MenC	Anti-MenW	Anti-MenY
1	356 \pm 2.5	310 \pm 2	176 \pm 4	479 \pm 1
2	2	996 \pm 1	2	2
3	10 \pm 8	11 \pm 4	4 \pm 5	34 \pm 2
4	2	3 \pm 3	2	2
5	81 \pm 3	54 \pm 3	22 \pm 2	162 \pm 2
6	10	246 \pm 2	7	8
7	21 \pm 2	42 \pm 2	11 \pm 3	79 \pm 1
8	2	94 \pm 4	2	2
9	140 \pm 4	103 \pm 4	118 \pm 2	285 \pm 2
10	2	205 \pm 1	2	2

The same animals were tested for serum bactericidal antibodies in the presence of baby rabbit complement. Strains used were A-F6124, C-C11, W135-5554 and Y-240539

Results were as follows (Figure 3):

#	Anti-MenA	Anti-MenC	Anti-MenW	Anti-MenY
1	512	1024	2048	8192
2	—	8192	—	—
3	64	128	96	8192
4	—	64	—	—
5	256	1024	1024	8192
6	—	4096	—	—
7	128	256	48	8192
8	—	512	—	—
9	2048	4096	1024	8192
10	—	2048	—	—

Proliferation of cells in the spleen was also tested for the same 10 groups. Results for odd-numbered groups, which received MenACWY antigens, are shown in Figure 4A; even-numbered groups, which received MenC only, are in Figure 4B.

In a second set of experiments, mice received 20 μ l of the following ACWY compositions (each antigen as 2 μ g saccharide) intranasally, except for group 1 which received it subcutaneously:

#	Adjuvant	Adjuvant (μ g)
1	Alum (s.c.)	500
2	Alum (i.n.)	500
3	LTK63	1
4	TMC	61
5	TMC	122
6	LTK63 + TMC	1 + 61
7	LTK63 + TMC	1 + 122
8	Chitosan	61
9	Chitosan	122
10	LTK63 + chitosan	1 + 61
11	LTK63 + chitosan	1 + 122

Serum IgG after three immunisations are shown in Figure 5, serum BCA are shown in Figure 6, and cell proliferation is shown in Figures 7A & 7B.

In a third set of similar experiments, mice received 20 μ l of the following ACWY compositions (each antigen as 2 μ g saccharide) intranasally, except for group 1 which received it subcutaneously:

#	Adjuvant	Adjuvant (μ g)
1	Alum (s.c.)	500
2	—	—
3	LTK63	1
4	LTK63	0.1
5	TMC	61
6	LTK63 + TMC	1 + 61
7	LTK63 + TMC	0.1 + 61
8	Chitosan	61
9	LTK63 + Chitosan	1 + 61
10	LTK63 + Chitosan	0.1 + 61

Serum IgG after three immunisations are shown in Figure 8, serum BCA are shown in Figure 9, and cell proliferation is shown in Figures 10A & 10B.

Thus both LTK63 and TMC, and particularly the pairing thereof, are highly effective adjuvants for intranasal delivery of a combined vaccine against meningococcal serogroups A, C, W135 and Y.

- 5 It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

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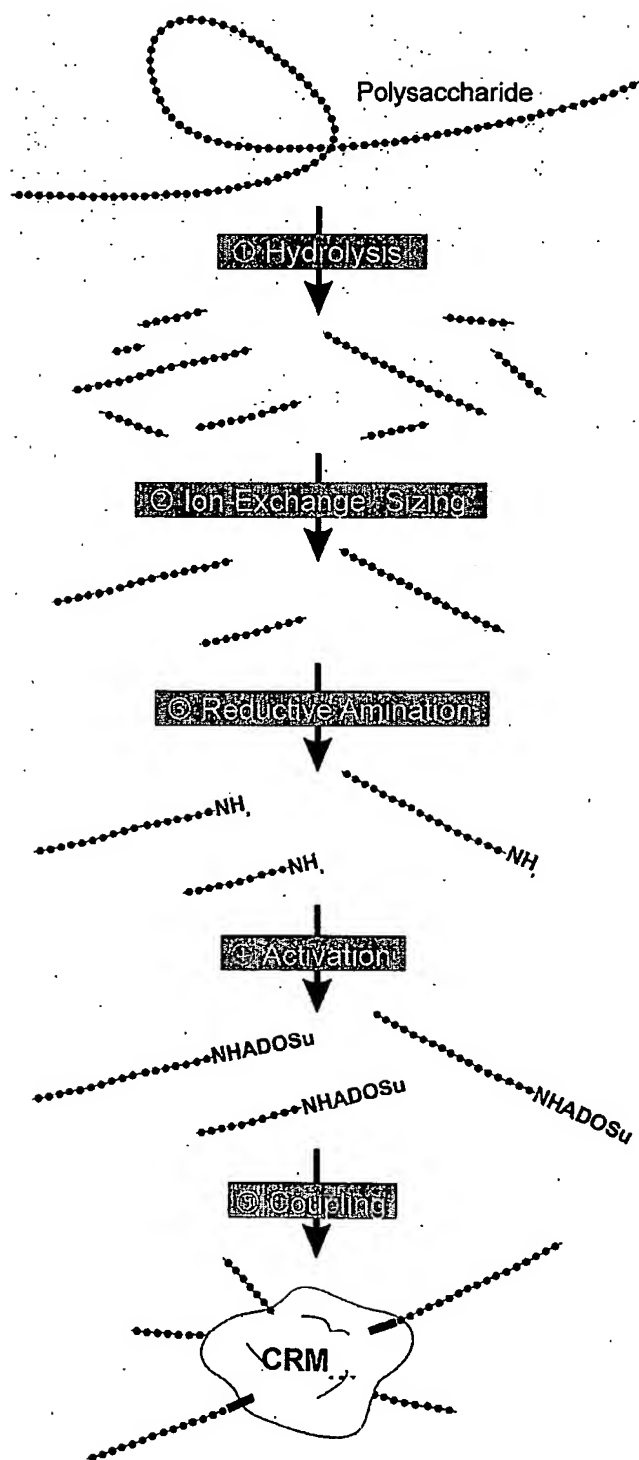
CLAIMS

1. An immunogenic composition for mucosal delivery, comprising capsular saccharides from at least two of serogroups A, C, W135 and Y of *N.meningitidis*.
2. An immunogenic composition, comprising (a) a capsular saccharide antigen from serogroup C of *N.meningitidis*, and (b) a chitosan adjuvant.
3. The composition of claim 2, comprising (c) one or more further antigens and/or (d) one or more further adjuvants.
4. The composition of any preceding claim, wherein the capsular saccharides are conjugated to carrier protein(s) and/or are oligosaccharides.
5. The composition of claim 3, wherein the capsular saccharides are oligosaccharides conjugated to carrier protein(s).
6. The composition of any preceding claim, comprising capsular saccharides from 2, 3 or 4 of serogroups A, C, W135 and Y of *N.meningitidis*.
7. The composition of claim 6, comprising saccharides from serogroups A+C, A+W135, A+Y, C+W135, C+Y, W135+Y, A+C+W135, A+C+Y, C+W135+Y, or A+C+W135+Y.
8. The composition of any preceding claim, which is adapted and/or packaged for intranasal administration.
9. The composition of claim 8, in the form of a nasal spray or nasal drops.
10. The compositions of any preceding claim, comprising a chitosan adjuvant and/or a detoxified mutant of *E.coli* heat-labile toxin.
11. The composition of claim 10, wherein the chitosan is a tri-alkylated chitosan.
12. The composition of claim 11, wherein the chitosan is a trimethylchitosan.
13. The composition of any one of claims 10 to 12, wherein the detoxified mutant of *E.coli* heat-labile toxin has a serine-to-lysine substitution at residue 63.
14. The composition of any preceding claim, wherein the composition does not include all three of (1) a meningococcal saccharide, (2) an antigen which induces an immune response against *Haemophilus influenzae*, and (3) an antigen which induces an immune response against *Streptococcus pneumoniae*.
15. The composition of any preceding claim, comprising all three of (1) a meningococcal saccharide, (2) an antigen which induces an immune response against *Haemophilus influenzae*, and (3) an

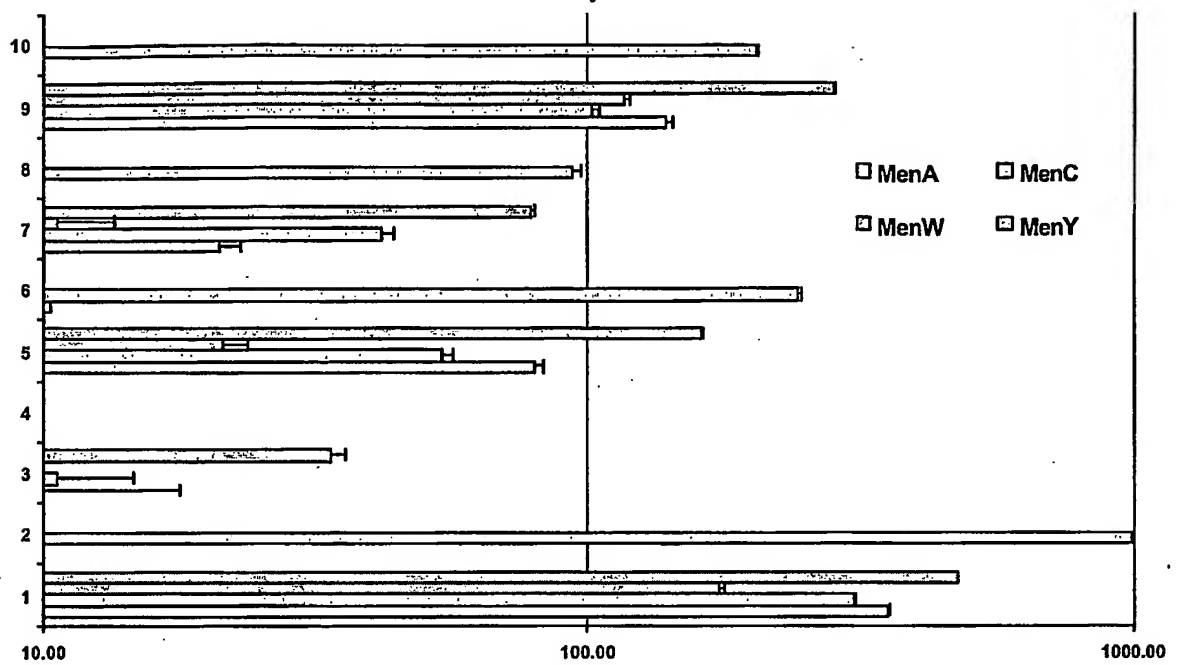
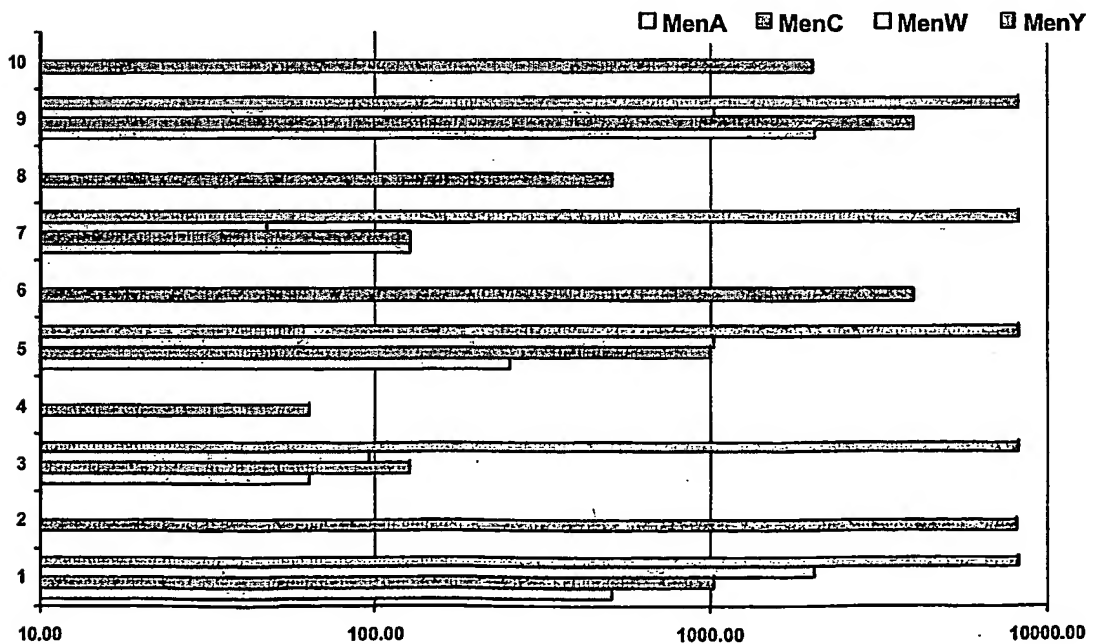
antigen which induces an immune response against *Streptococcus pneumoniae*, and an alkylated derivative of chitosan.

16. A kit comprising: (a) capsular saccharide from *N.meningitidis* serogroup A, in lyophilised form; and (b) capsular saccharide(s) from one or more of *N.meningitidis* serogroups C, W135 and Y, in liquid form, wherein (a) and (b) are formulated such that, when combined, they are suitable for mucosal administration.
17. The invention provides a method of raising an immune response in a patient, comprising administering to a patient a composition according to any one of claims 1 to 15.
18. Use of capsular saccharides from at least two of serogroups A, C, W135 and Y of *N.meningitidis*, wherein said capsular saccharides are conjugated to carrier protein(s) and/or are oligosaccharides, in the manufacture of a medicament for mucosal delivery to an animal in order to raise an immune response.
19. Use of (1) a capsular saccharide from at least one of serogroups A, C, W135 and Y of *N.meningitidis*, wherein said capsular saccharides are conjugated to carrier protein(s) and/or are oligosaccharides, and (2) a chitosan, in the manufacture of a medicament for mucosal delivery to an animal in order to raise an immune response.
20. Use of claim 18 or claim 19, wherein the medicament is for intranasal delivery.
21. A vaccine composition comprising a chitosan adjuvant, a mutant ADP-ribosylating toxin and an antigen, wherein the vaccine composition gives a Th1-biased immune response after administration to a subject.

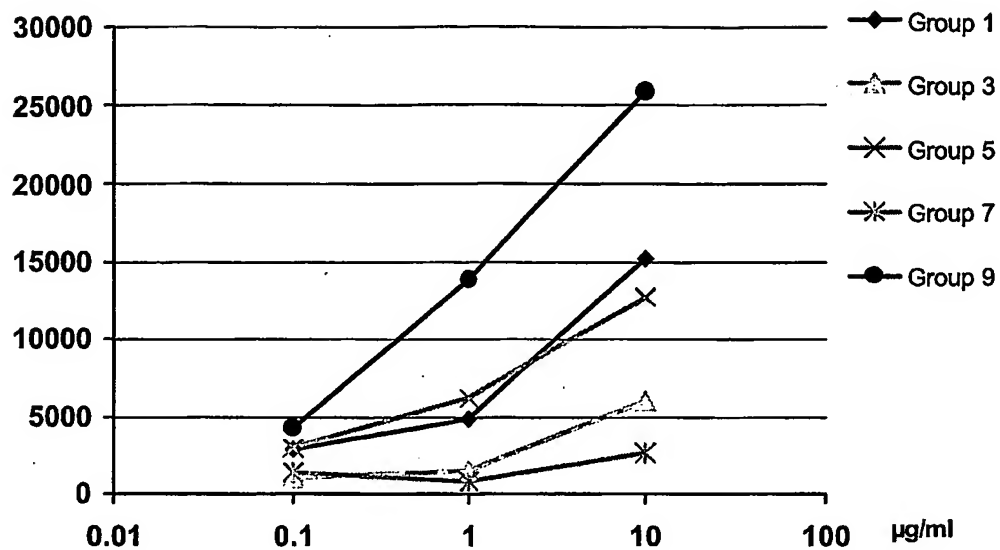
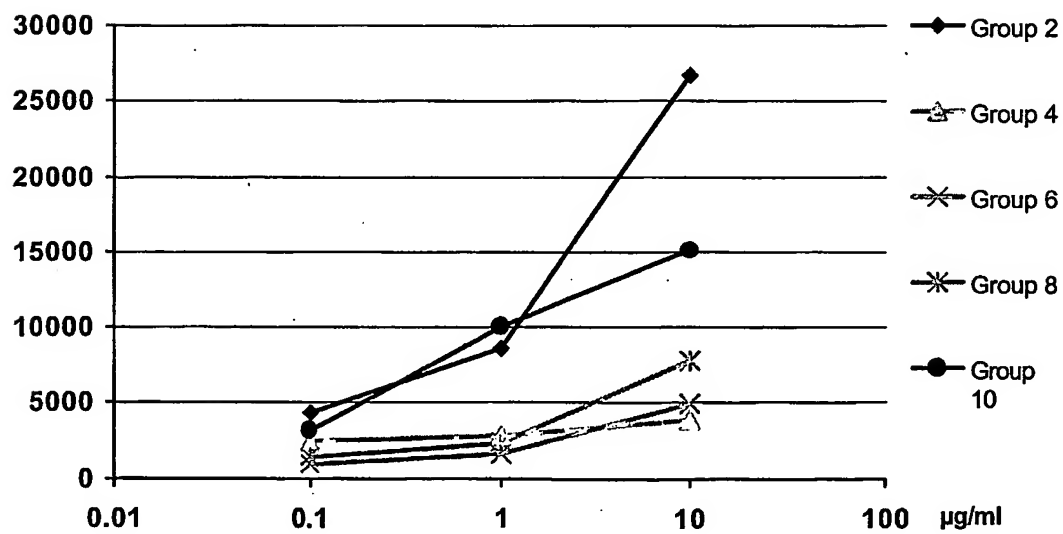
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FIGURE 1

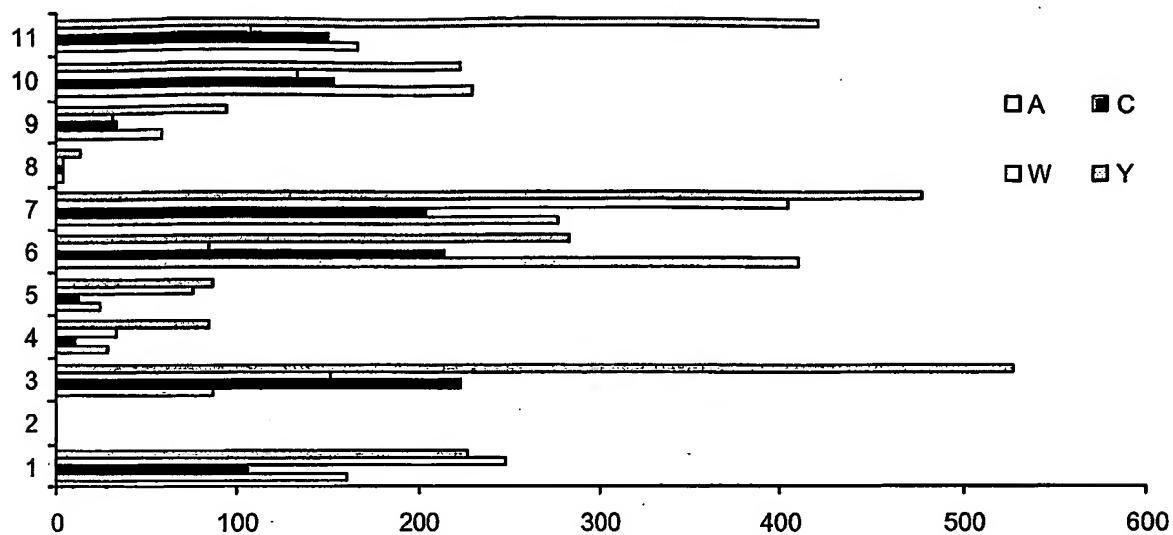
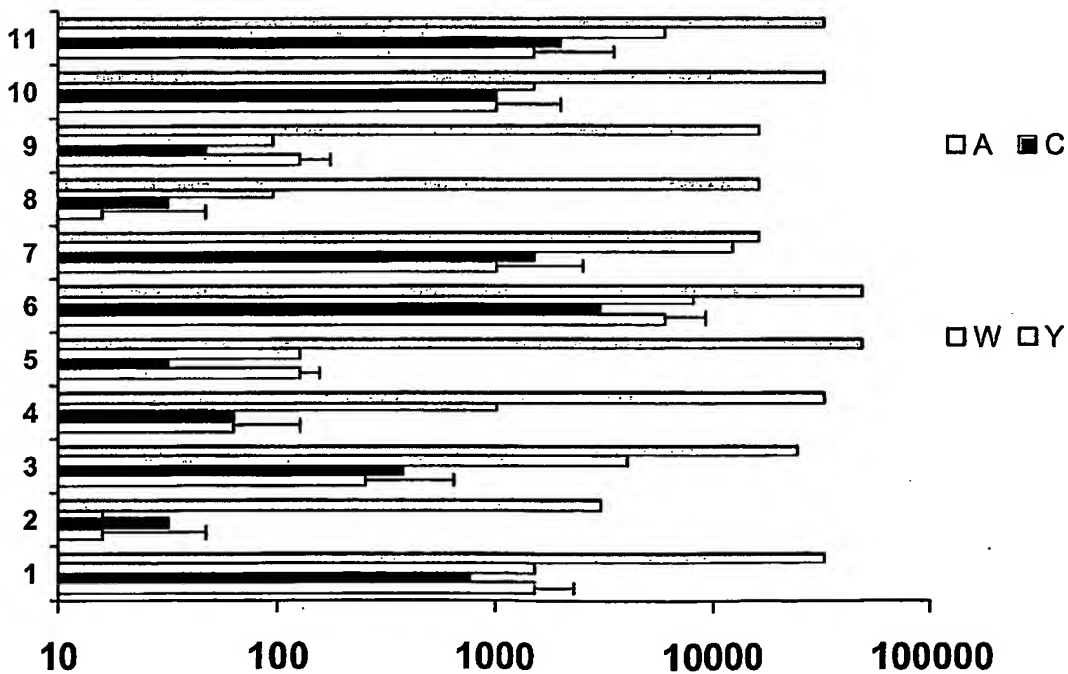
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FIGURE 2**FIGURE 3**

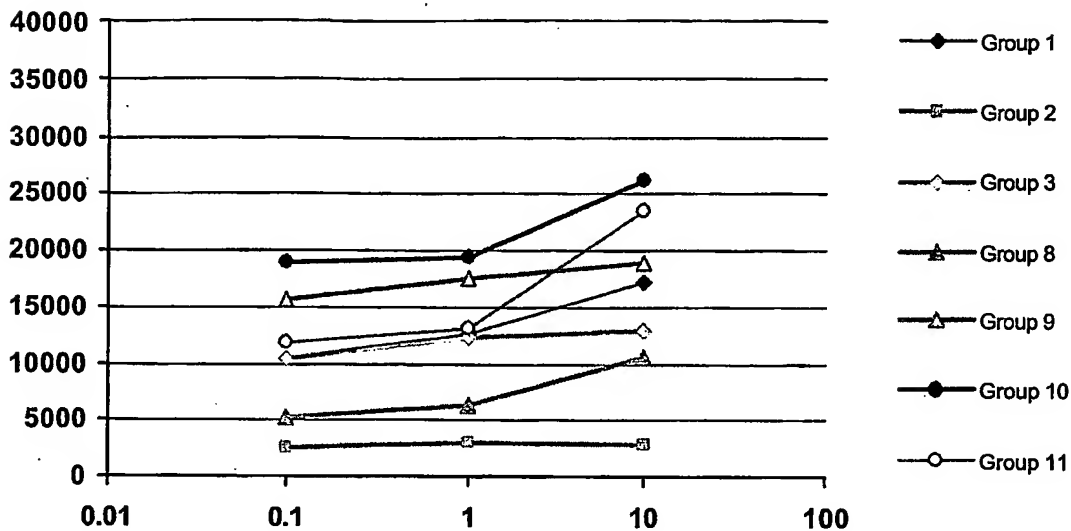
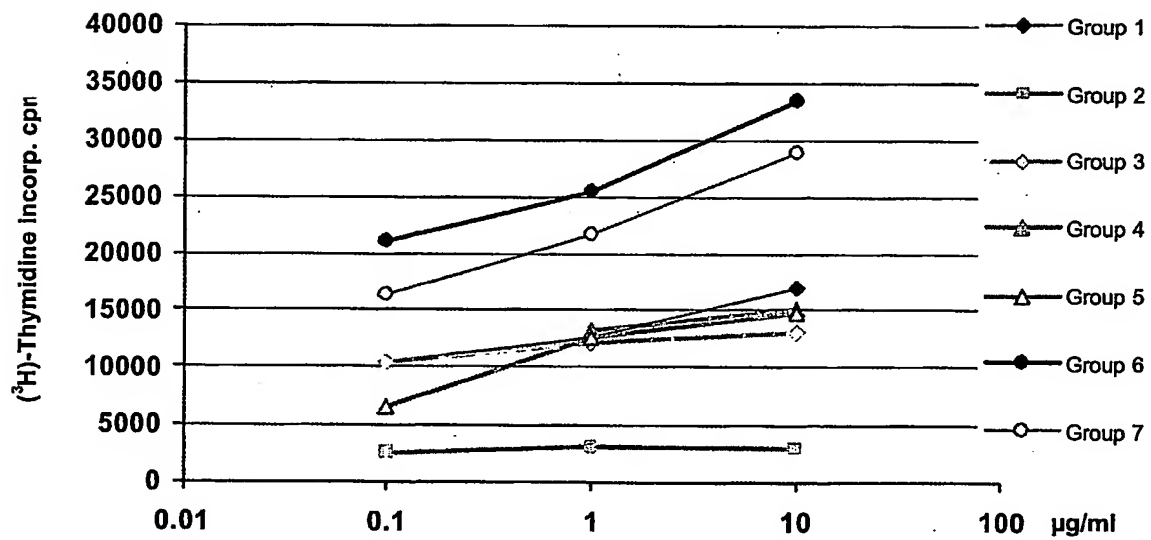
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FIGURE 4A**FIGURE 4B**

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FIGURE 5**FIGURE 6**

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FIGURE 7A**FIGURE 7B**

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FIGURE 8

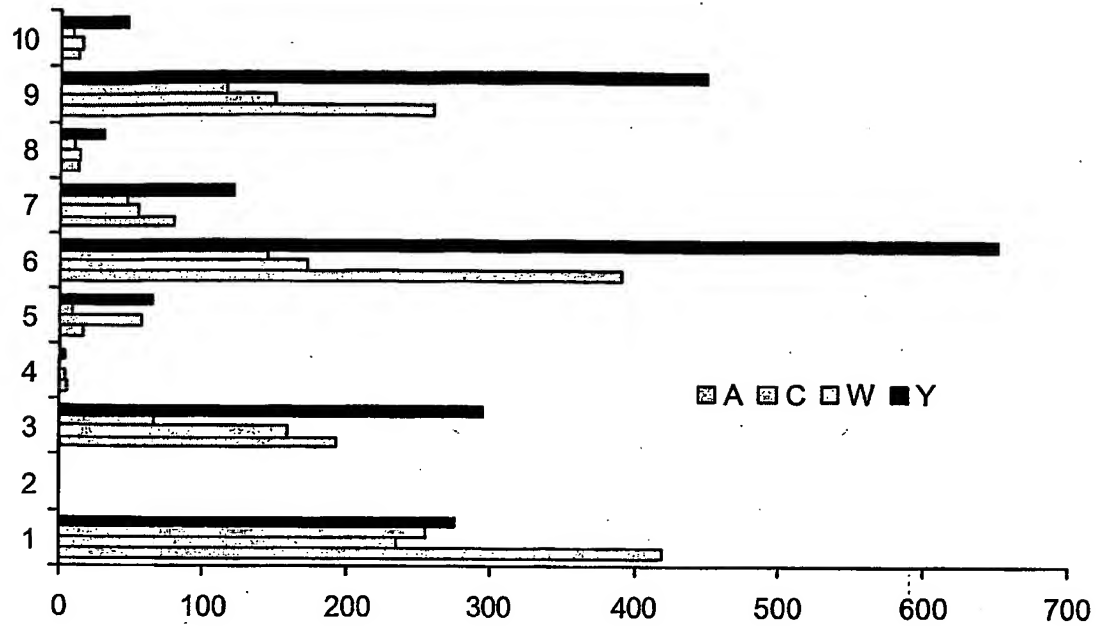
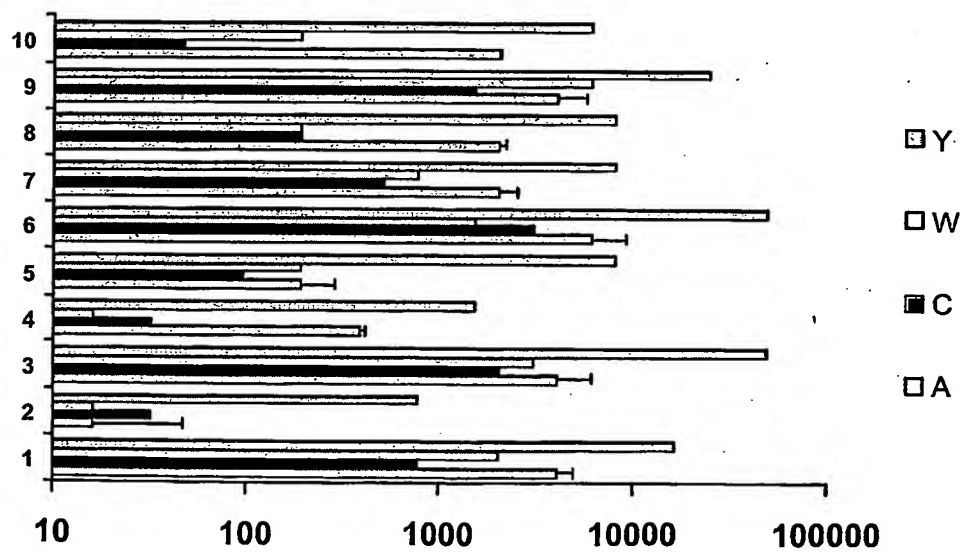
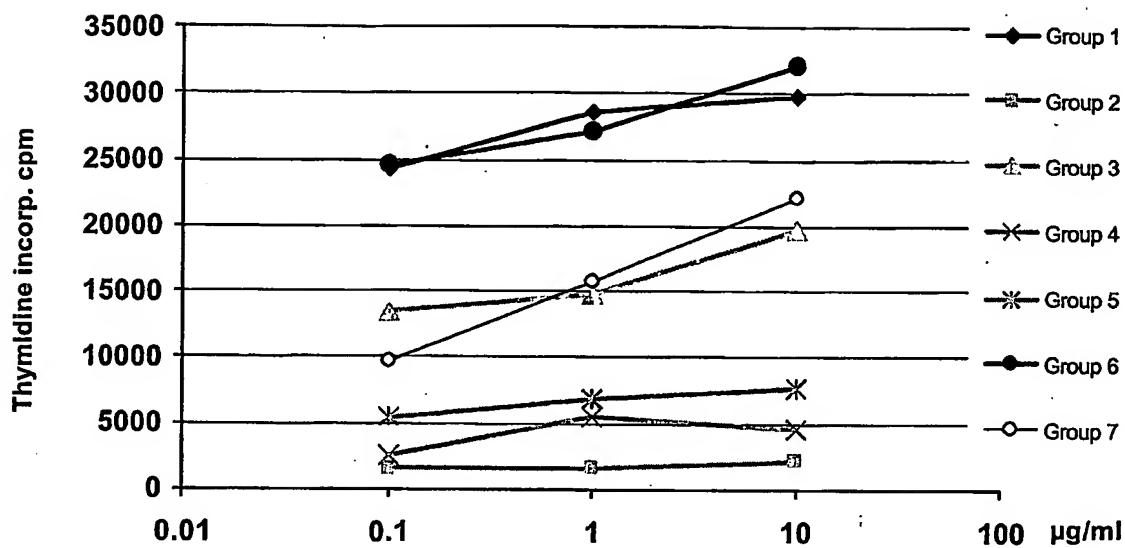
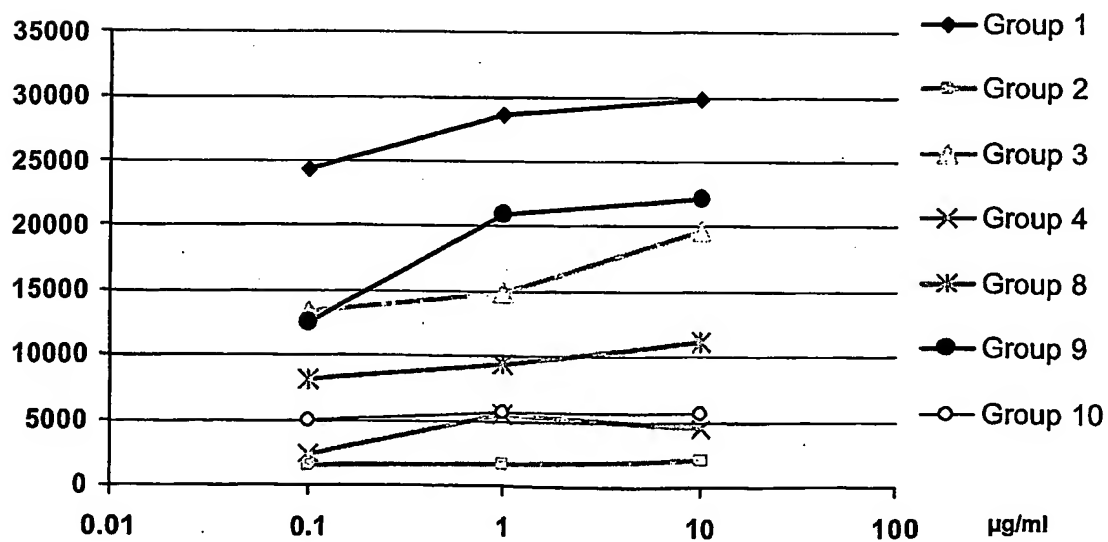


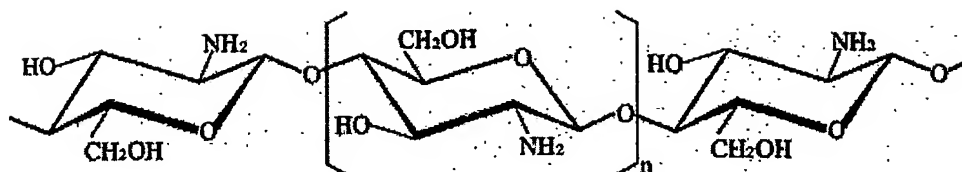
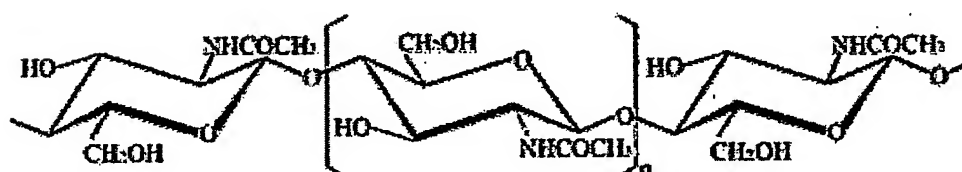
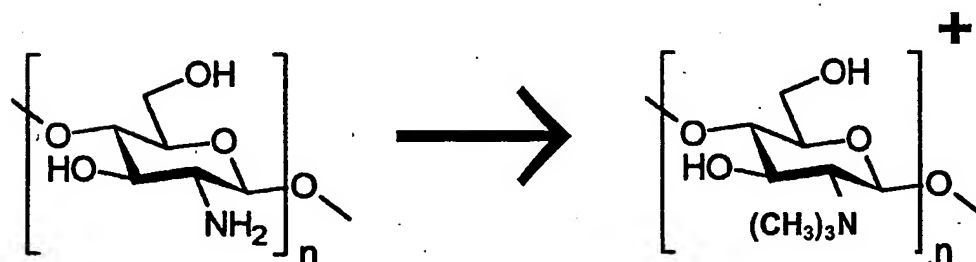
FIGURE 9



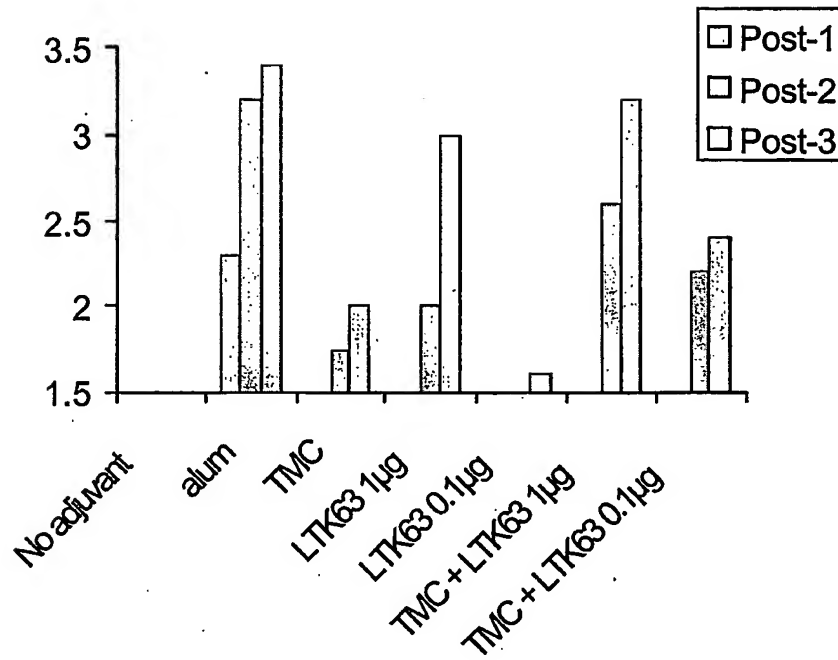
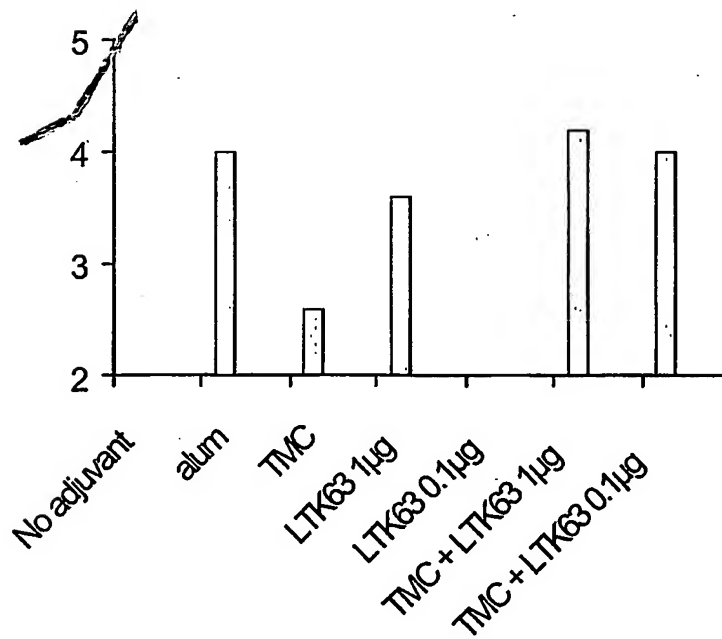
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FIGURE 10A**FIGURE 10B**

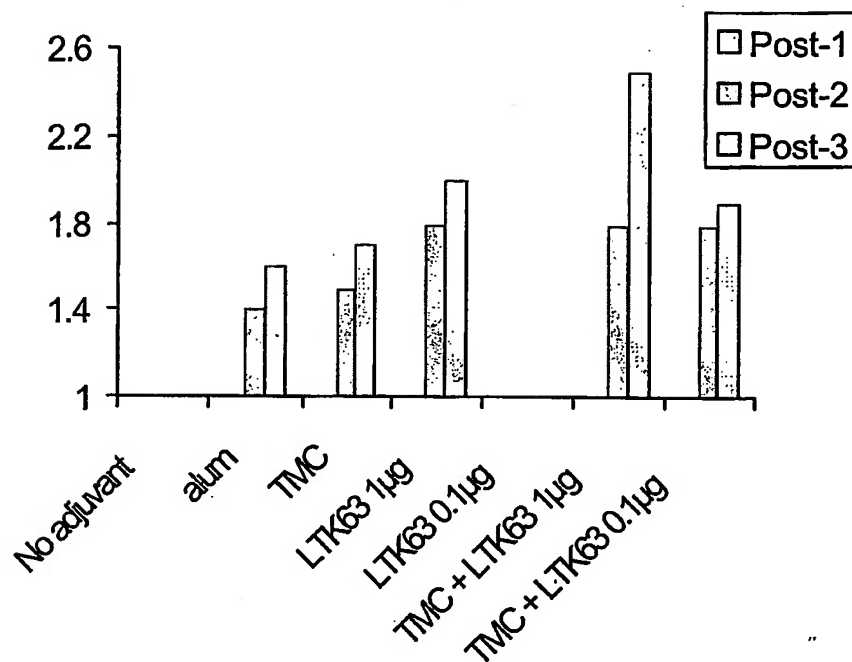
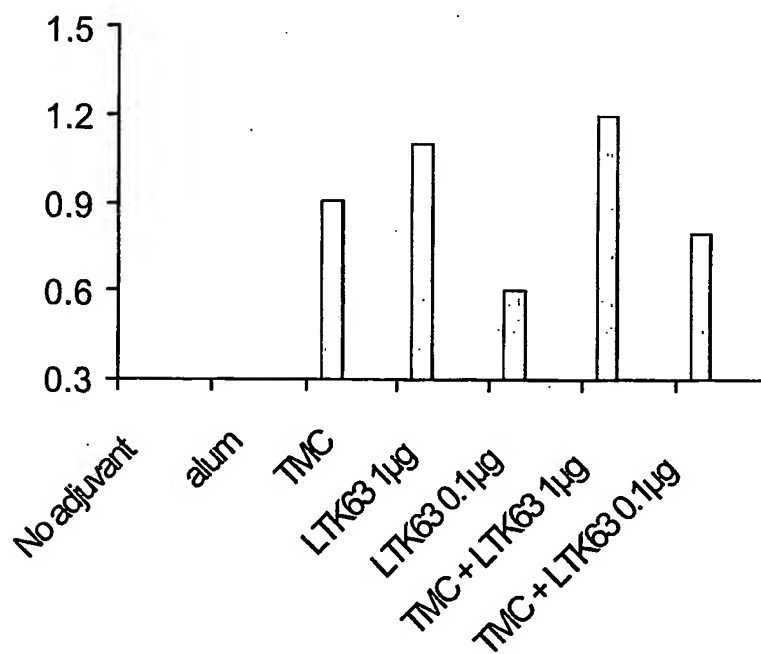
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FIGURE 11**FIGURE 12****FIGURE 13**

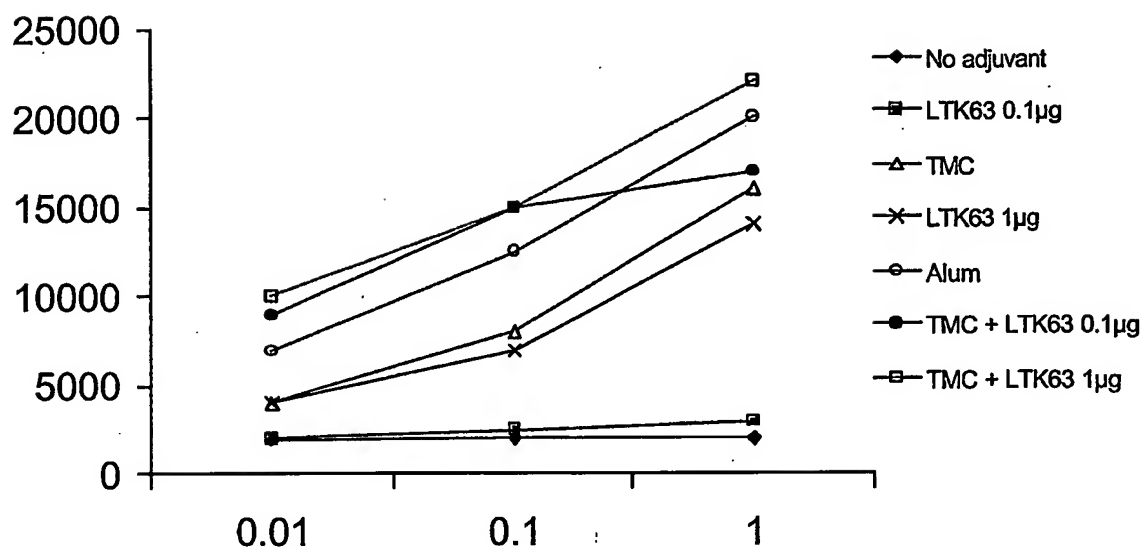
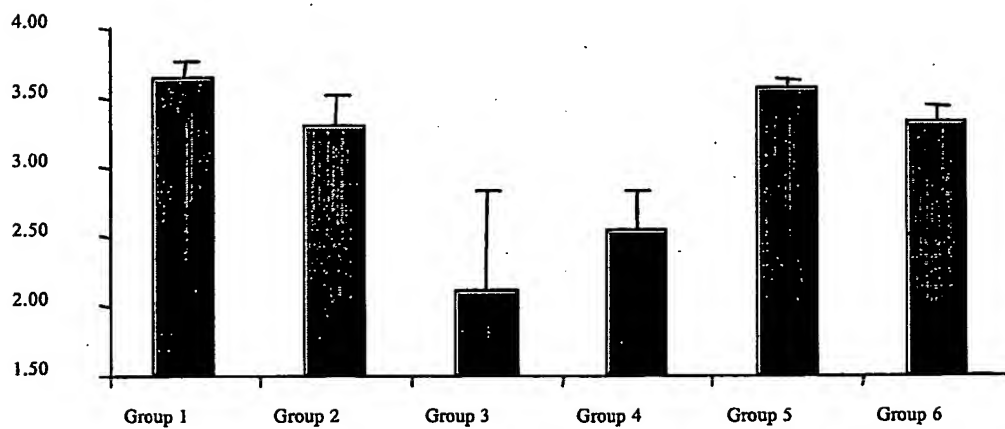
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FIGURE 14A**FIGURE 14B**

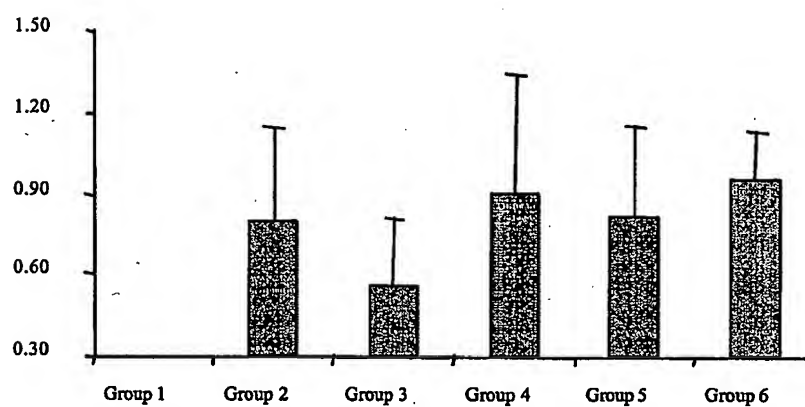
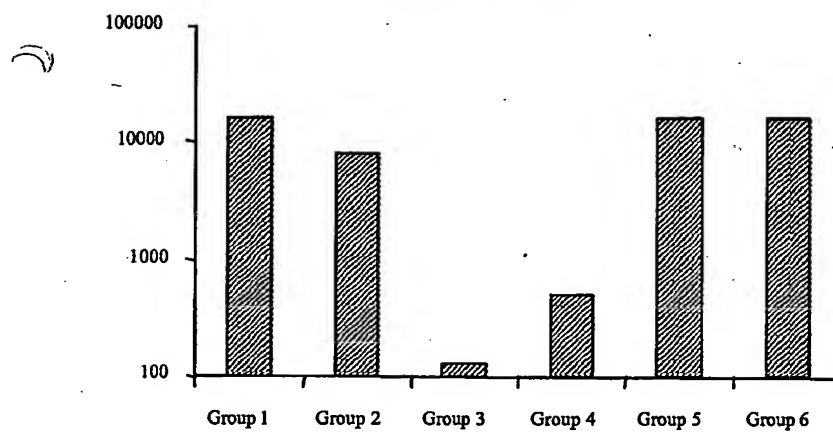
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FIGURE 15A**FIGURE 15B**

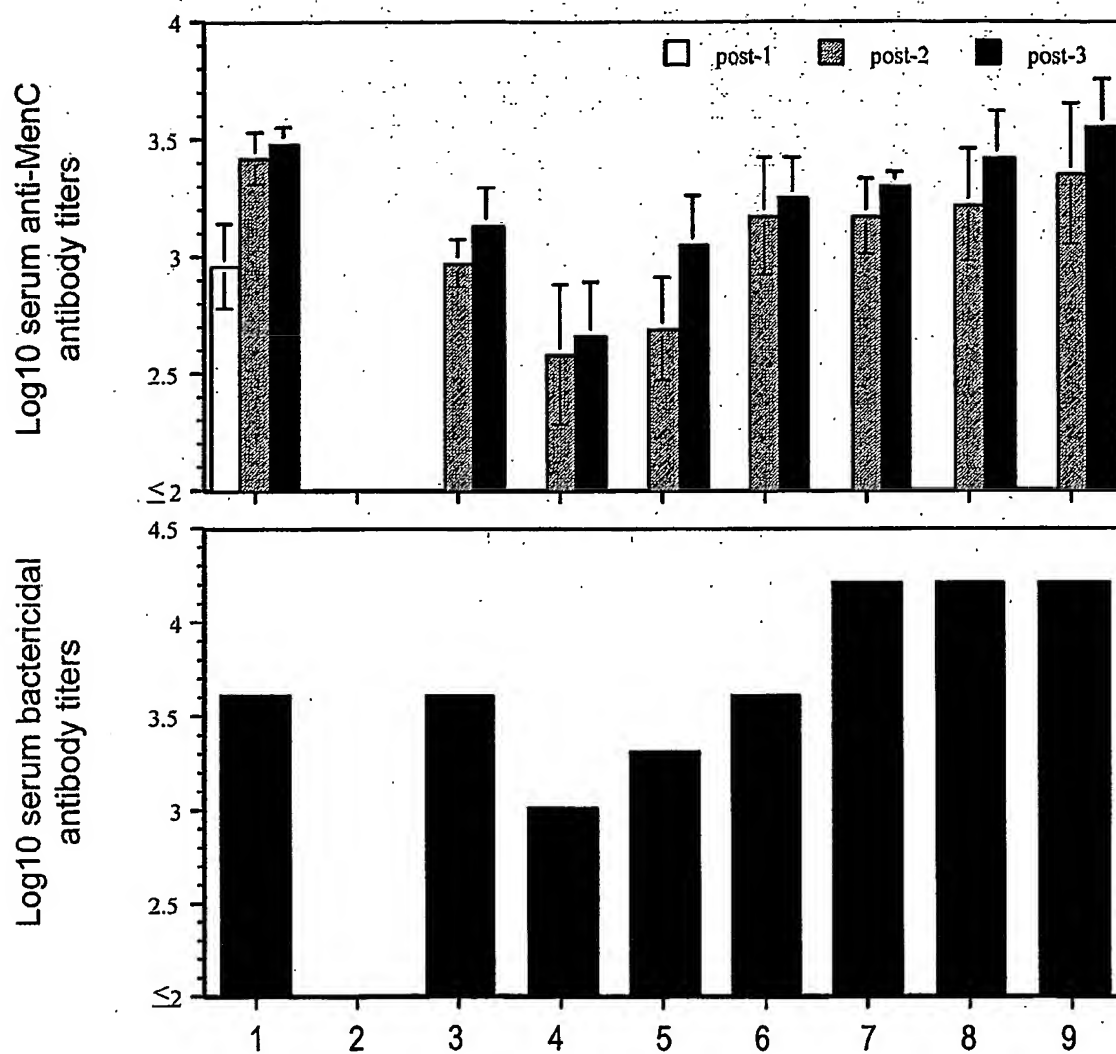
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FIGURE 16**FIGURE 17**

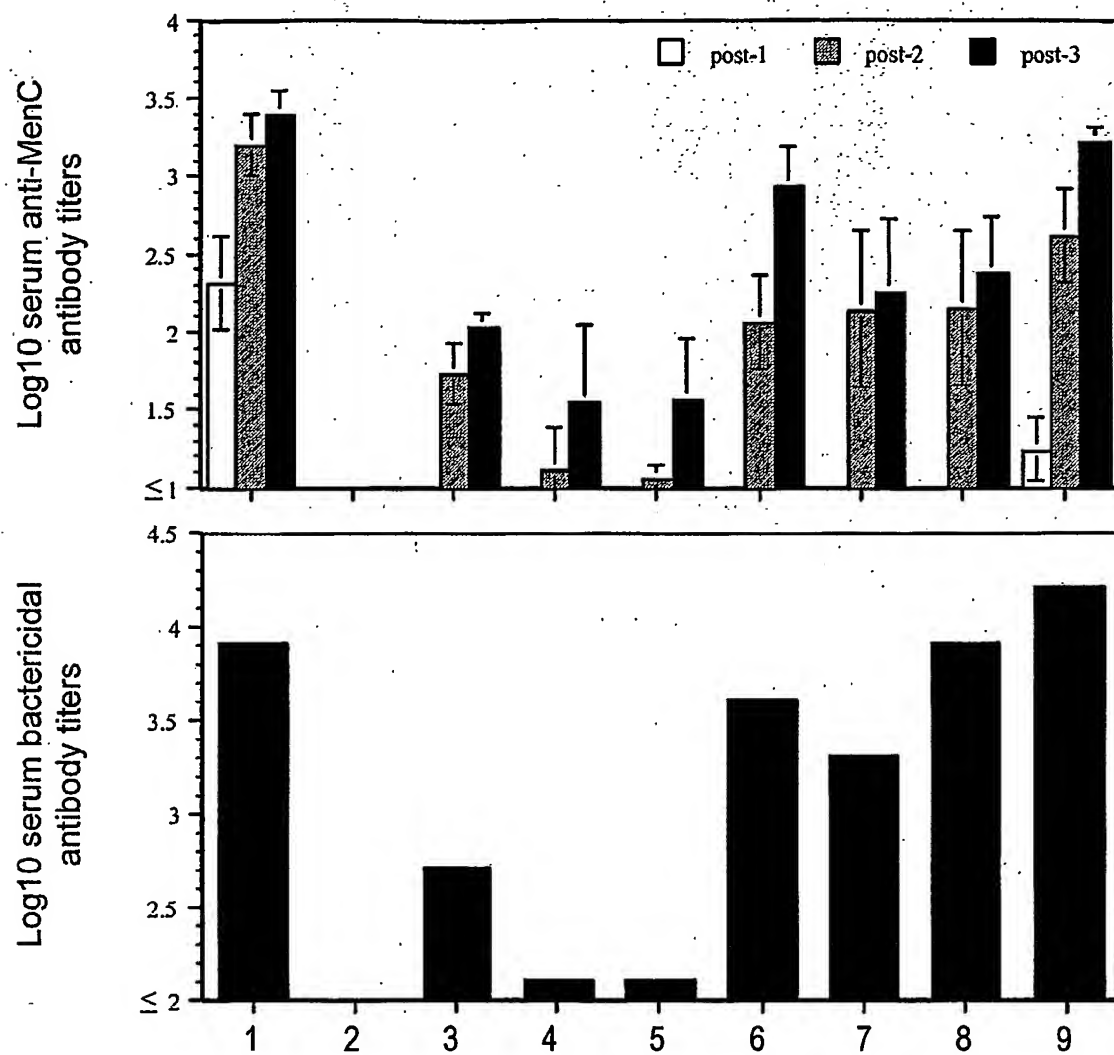
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FIGURE 18**FIGURE 19**

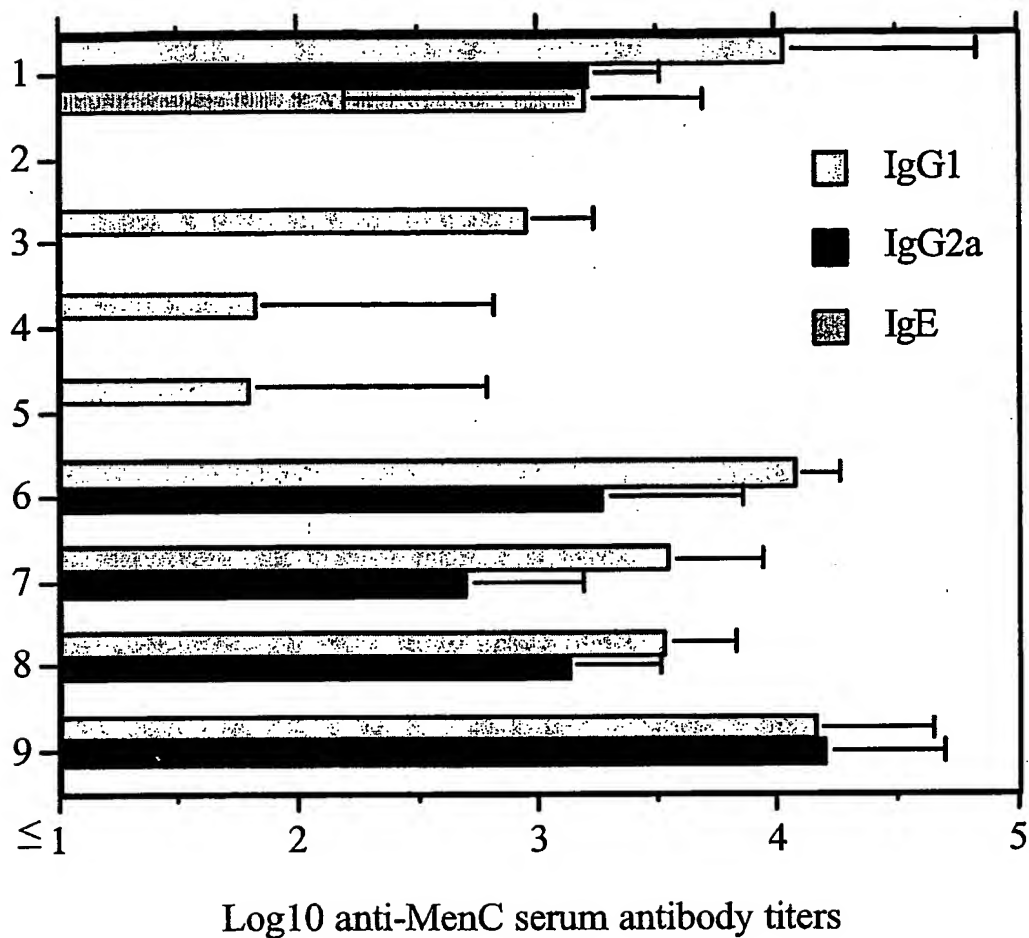
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FIGURE 20

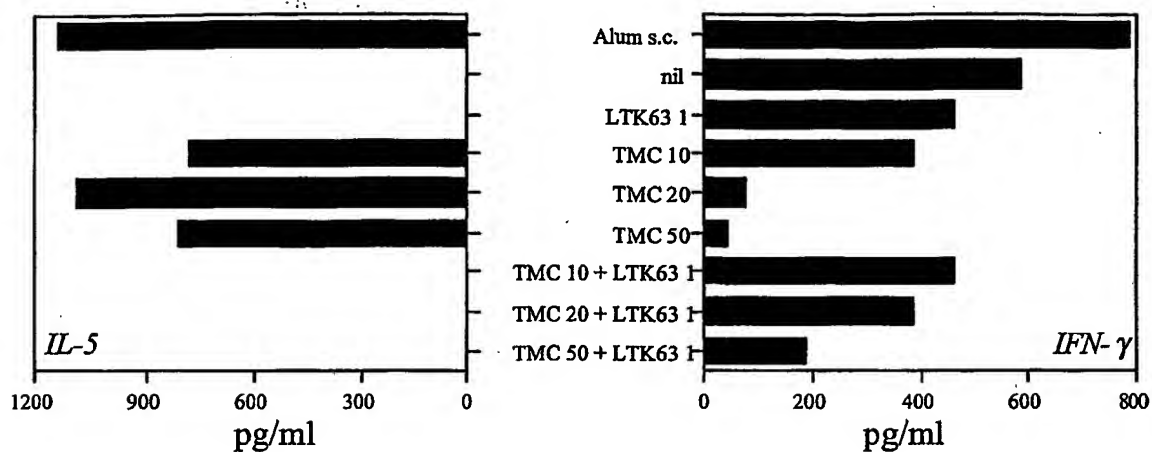
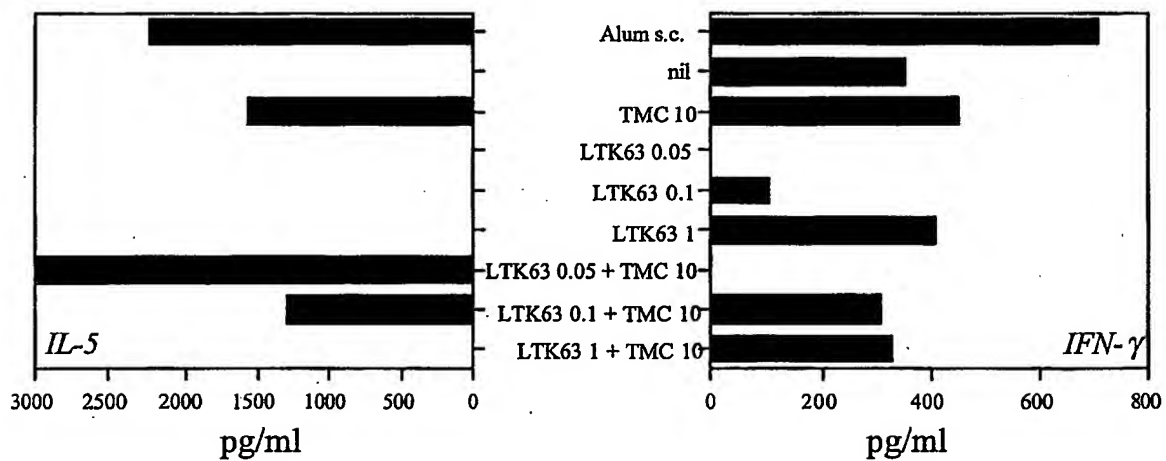
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FIGURE 21

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FIGURE 22

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FIGURE 23**FIGURE 24**

INTERNATIONAL SEARCH REPORT

In **1**al Application No
PC1/1B2004/000673

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 A61K39/385 A61K39/095 A61K39/02 A61K39/102 A61K39/09		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the International search (name of data base and, where practical, search terms used) BIOSIS, EPO-Internal, EMBASE, MEDLINE, WPI Data, PAJ		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/00249 A (DESMONS PIERRE MICHEL ;LEMOINE DOMINIQUE (BE); SMITHKLINE BEECHAM) 3 January 2002 (2002-01-03) cited in the application page 1, line 22 -page 2, line 3 page 2, line 30 -page 3, line 2 page 3, line 23 - line 29 page 5, line 23 page 6, line 4 - line 8 page 8, line 18 - line 25	1,4,6-8, 11,12, 14, 16-18,20
Y	the whole document	15
X	WO 99/42130 A (CONNAUGHT LAB ;CHONG PELE (CA); KLEIN MICHEL H (CA); LINDBERG ALF) 26 August 1999 (1999-08-26) cited in the application claims 2,6,7 page 31, line 17 - line 22	1,4,6,8, 9,14,17, 18,20
-/--		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		
<input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents :		
A document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed		
T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *Z* document member of the same patent family		
Date of the actual completion of the international search 7 June 2004		Date of mailing of the international search report 21/06/2004
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Irion, A

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International Application No
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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	<p>WO 02/077021 A (CHIRON SPA ;MASIGNANI VEGA (IT); FRASER CLAIRE (US); TETTELIN HERV) 3 October 2002 (2002-10-03) the whole document</p>	1-20
A	<p>MCNEELA E A ET AL: "A mucosal vaccine against diphtheria: formulation of cross reacting material (CRM197) of diphtheria toxin with chitosan enhances local and systemic antibody and Th2 responses following nasal delivery" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 19, no. 9-10, 8 December 2000 (2000-12-08), pages 1188-1198, XP004227926 ISSN: 0264-410X cited in the application the whole document</p>	1-21
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB2004/000673

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>RYAN ELIZABETH J ET AL: "Mutants of Escherichia coli heat-labile toxin act as effective mucosal adjuvants for nasal delivery of an acellular pertussis vaccine: Differential effects of the nontoxic AB complex and enzyme activity on Th1 and Th2 cells"</p> <p>INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 67, no. 12, December 1999 (1999-12), pages 6270-6280, XP002162796</p> <p>ISSN: 0019-9567</p> <p>the whole document</p>	1-21
A	<p>RYAN E J ET AL: "Modulation of innate and acquired immune responses by Escherichia coli heat-labile toxin: distinct pro- and anti-inflammatory effects of the nontoxic AB complex and the enzyme activity."</p> <p>JOURNAL OF IMMUNOLOGY (BALTIMORE, MD.: 1950) UNITED STATES 15 NOV 2000, vol. 165, no. 10, 15 November 2000 (2000-11-15), pages 5750-5759, XP002283476</p> <p>ISSN: 0022-1767</p> <p>cited in the application</p> <p>the whole document</p>	1-21
P,X	<p>WO 03/007985 A (CHIRON SPA ;COSTANTINO PAOLO (IT)) 30 January 2003 (2003-01-30)</p> <p>cited in the application</p> <p>the whole document</p>	1-20
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T	<p>BAUDNER BARBARA C ET AL: "The concomitant use of the LTK63 mucosal adjuvant and of chitosan-based delivery system enhances the immunogenicity and efficacy of intranasally administered vaccines."</p> <p>VACCINE, vol. 21, no. 25-26, 8 September 2003 (2003-09-08), pages 3837-3844, XP004446158</p> <p>ISSN: 0264-410X (ISSN print)</p> <p>the whole document</p>	1-20

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International application No.
PCT/IB2004/000673

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 17 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

 International Application No
 PCT/IB2004/000673

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